Characterization of the *Arabidopsis* Family of *PURINE PERMEASES* as Candidates for a Cytokinin Transport System

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Abstract

Plant development is characterized by stereotypic growth and patterning processes and by the extraordinary capability of plants to continuously grow and produce new organs throughout their life. Growth and developmental plasticity depend on intricate regulatory machineries that are often coordinated by hormones. Cytokinins constitute one class of these essential growth factors. They trigger the activation of a multistep two-component phosphorelay system that results in altered expression of target genes. The sites and stages at which phosphorelay signalling occurs are precisely specified. Although regulated cytokinin biosynthesis, modification and degradation are crucial determinants in the control of local cytokinin levels, they alone cannot account for the spatiotemporal definition of cytokinin signalling domains observed *in planta*. Rather, the evidence supports that cytokinins are differentially distributed within the plant and tissues, thereby establishing defined cytokinin signalling landscapes. Members of the *Arabidopsis* gene family of *PURINE PERMEASES* (*PUPs*) have been identified as importers of cytokinins and other adenine-derived substrates and might therefore constitute mediators of cytokinin short-range transport. To test this hypothesis, this thesis aimed at the characterization of the *PUP* family trying to identify the relevant members involved in shaping cytokinin signalling domains.

We established an expression profile of the 23 *PUP* family members in *Arabidopsis*, and identified *PUP14* as most abundantly and ubiquitously expressed member. With the aid of the synthetic sensor *TCSn::GFP* that reports and visualizes live cytokinin signalling output, we found that the inducible loss of *PUP14* causes ectopic cytokinin signalling suggesting that restriction of signalling is dependent on *PUP14* function. *PUPs* encode trans-membrane proteins and PUP14 is targeted to the plasma membrane. Its expression pattern in the embryo, shoot and root apical meristem, lateral root, and female gametophyte exhibits complementarity to the cytokinin signalling domains and we show that the expression of the protein in protoplasts is correlated with an increased cytokinin import rate. Together, these data indicate that *PUP14* maintains cytokinin signalling patterns by the import of active cytokinin thereby prohibiting signalling in these cells. This implies a central role for apoplastic cytokinins in initiating phosphorelay via plasma membrane-localized receptors, ARABIDOPSIS HISTIDINE KINASES, which were previously reported to reside predominantly in the membranes of the endoplasmic reticulum.

By the use of an RNAi approach and the assessment of expression patterns and transport activities, we determined that the function of *PUP14* is resumed in other but not all analysed *PUPs*. Downregulation of a group of less strongly expressed *PUP* members results in ectopic cytokinin signalling output in the embryo. Moreover, members *PUP4* and *PUP11* also show expression patterns that are complementary to cytokinin signalling in specific contexts. In contrast, the expression pattern and transport capacity of PUP18 suggest that this family member does not act as a cytokinin sink; *PUP18* is expressed in epidermal

tissue and only shows weak stimulation of cytokinin import in protoplasts. Overexpression assays furthermore indicate that additional, yet uncharacterized mechanisms operate to ensure stable cytokinin signalling domains required for plant development.

Zusammenfassung

Pflanzenwachstum zeichnet sich aus durch stereotypische Wachstums- und Musterbildungsprozesse und durch die aussergewöhnliche Fähigkeit von Pflanzen, ein Leben lang zu wachsen und neue Organe zu bilden. Wachstum und auch Entwicklungsplastizität sind abhängig von komplexen regulatorischen Abläufen, welche von Hormonen koordiniert werden. Zytokinine repräsentieren eine Klasse solcher kritischen Wachstumsfaktoren. Zytokinine verändern die Expression von Zielgenen durch die Initiierung eines Phosphotransfer- Signalwegs, dessen Aktivität zeitlich und räumlich präzis definierten wird. Obwohl regulierte Biosynthese, Modifikation und der Abbau von Zytokininen elementar sind, um die Mengen lokal verfügbaren Zytokinins zu kontrollieren, reichen diese Prozesse nicht aus, um die beobachteten Aktivitätsmuster zu definieren. Vielmehr scheint es, dass Zytokinine in der Pflanze und in Organen unterschiedlich verteilt werden, damit solche Aktivitätsfelder beschrieben werden können. Die Forschung hat erkannt, dass einzelne Proteine der Familie der Purin Permeasen in *Arabidopsis* die Fähigkeit zeigen, Zytokinine und andere Derivate von Adenin zu importieren, weshalb sie möglicherweise Kandidaten sind, um die gezielte Zytokininverteilung im Gewebe zu bewerkstelligen. Das Ziel der folgenden Dissertation war es daher, die Genfamilie der Purin Permeasen zu charakterisieren, und dadurch diejenigen Purin Permeasen zu identifizieren, die für das Kreieren der Zytokininaktivitätsmuster notwendig sind.

Wir haben ein Expressionsprofil der 23 Purine Permease Genen in *Arabidopsis* erstellt und entdeckt, dass die Purin Permease 14 das am häufigsten vorkommende Familienmitglied ist. Mit Hilfe des künstlich entwickelten Reporterkonstrukts TCSn haben wir feststellen können, dass die herbeigeführte Runterregulierung des Purin Permease 14 Gens zu einer Erweiterung der Regionen führt, in denen Zytokininsignalwege aktiv sind. Dies bedeutet, dass die Beschränkung der Zytokininfeldern von funktionierenden Purin Permeasen 14 abhängig ist. Purin Permeasen sind Proteine mit membranumspannenden Domänen und Purin Permeasen 14 befinden sich in der Plasmamembran. Das Expressionsmuster der Purin Permease 14 in Embryonen, Apikalmeristemen, Seitenwurzeln und weiblichem Gametophyt ist negativ korreliert mit den Aktivitätsfeldern von Zytokinin. Im Weiteren konnten wir zeigen dass isolierte Pflanzenzellen, welche erhöhte Mengen der Purin Permease 14 aufweisen, auch eine erhöhte Menge von Zytokininen importieren. Diese Resultate deuten darauf hin, dass diese Purin Permease durch den Import von aktiven Zytokininen die Aktivitätsfelder definiert, woraus zu schliessen ist, dass apoplastische Zytokinine wesentlich sind für die Initiierung des intrazellulären Phosphotransfers. Dies impliziert wiederum, dass die Zytokininrezeptoren, ARABIDOPSIS HISTIDIN KINASEN, plasmamembranständig sind, obschon zuvor gezeigt werden konnte, dass diese überwiegend in der Membran des endoplasmatischen Retikulum zu finden sind.

Mittels RNA Interferenz, Analyse der Expressionsmuster und Messungen der Transportaktivitäten konnten wir erkennen, dass die Funktion, welche die Purin Permease

14 innehat, teils auch von anderen untersuchten Purin Permeasen wahrgenommen wird. Zeitgleiche Runterregulierung einer Gruppe seltener auftretenden Purin Permeasen führt im Embryo zu zusätzlichen Zytokininsignalfeldern. Ausserdem beschreiben auch die Purin Permeasen 4 und 11 Expressionsmuster, die komplementär zu Zytokininaktivitätsfeldern sind. Im Gegensatz dazu deuten das Expressionsmuster und die gemessene Transportkapazität von Purin Permease 18 darauf hin, dass dieses Protein eine andere Funktion hat, als die Zytokininverteilung zu etablieren; Purine Permease 18 kommt in der Epidermis vor und stimuliert den Import von Zytokinin nur schwach. Im Weiteren haben Überexpressionstests angedeutet, dass zusätzliche, noch nicht genauer beschriebene Mechanismen wirken, um die robusten Zytokininaktivitätsfelder zu formen.

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1 Introduction

Plants have the remarkable capability to "succeed in splitting the CO_2 on the earth's surface by the low-energy quanta of sunlight" (Warburg, 1964) in the process of photosynthesis. Therefore, life on earth ultimately depends on plants - as primary producers and for their production of O_2. But even the giant sequoia, which is represented by the world's largest single-stem tree, The General Sherman Tree (1486.6 m^3) (Sequoia National Park, 1997), has started life as a seed. To understand how a small seed can become such an impressive mature plant, researchers have been scrutinizing plant growth and development trying to identify the requirements that allow the progressions in the plant life cycle. Using more amenable species than the giant sequoia, phytohormones were found to be instrumental in guiding the growth and patterning processes that determine morphology. Amongst these phytohormones, cytokinins constitute one well-described class of growth regulators. Commonly known phenomena, such as the occurrence of witches' broom, bushy growth of houseplants after trimming, or crown gall tumour formation are partially mediated by cytokinin action. Many other, more intricate processes are dependent on regulatory networks involving cytokinins, and will be explained below.

The following detailed introduction tries to underpin the importance of cytokinins and revisits the current knowledge in the field of cytokinin biosynthesis, signalling, metabolism and functions. A later section is devoted to the presentation of recent updates that deepen our understanding of cytokinin-governed processes. In the last introductory section, I will present a more detailed summary of known modes of cytokinin transport in order to emphasize the importance of differential cytokinin translocation, which is the main object of this thesis.

The following chapter in its current form is in press as a book chapter in *International Review of Cell and Molecular Biology_324*.

1.1 CYTOKININ SYNTHESIS, SIGNALLING AND FUNCTION - ADVANCES AND NEW INSIGHTS

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ABSTRACT

The plant hormones referred to as cytokinins are chemical signals that control numerous developmental processes throughout the plant life cycle including gametogenesis, root meristem specification, vascular development, shoot and root growth, meristem homeostasis, senescence, and more. In addition, they mediate responses to environmental cues such as light, stress, or nutrient conditions. The core mechanistic of cytokinin metabolism and signalling has been elucidated, but more layers of regulation, additional functions, and interactions with other signals are continuously discovered and described. In this chapter, we recapitulate the highlights of over one hundred years of cytokinin research covering the isolation of cytokinin, the elucidation of phosphorelay signalling, and how cytokinin functions in various developmental contexts including its interaction with other pathways. Additionally, given cytokinin's paracrine signalling mechanism, we postulate that cellular exporters for cytokinins exist.

Key words: cytokinin, phosphorelay signalling, plant development, hormones

INTRODUCTION

Cytokinins represent a class of phytohormones that are commonly associated with youth, growth and health. This view is based on their ability to stimulate cell division and growth (Miller et al., 1955; Riou-Khamlichi et al., 1999), and to counteract senescence (Richmond and Lang, 1957; Engelbrecht et al., 1969). However, cytokinins also promote cell differentiation (Dello Ioio et al., 2008), and even cell death (Vescovi et al., 2012). These opposing roles illustrate that cytokinins' specific functions can only be defined within the developmental context they are acting in. Cytokinin's general function could be summarised as trigger of cellular change, essential for numerous decisions throughout the plant life cycle, which includes both developmental processes, and adaptive responses to a changing abiotic and biotic environment.

What mechanisms guarantee that selected cells change their functions in response to a cytokinin stimulus? On one hand, active ligands need to be in the right place at the right time. This involves cytokinin biosynthesis, transport, modifications, as well as degradation. On the other hand, a recipient cell has to express all of the necessary components involved in sensing and relaying the initial stimulus to change the gene expression programme in the nucleus. The specific response is further influenced by the cross talk with other signals that vary with the cell's history and context.

Since the discovery of the first cytokinin, tremendous amount of data has accumulated - therefore, it is inevitable that this chapter will be selective. To obtain a comprehensive overview of the field, the consultation of additional articles is recommended (Heyl et al., 2012; Hwang et al., 2012; El-Showk et al., 2013; Kieber and Schaller, 2014). Specifically, readers interested in cytokinin's role in mediating environmental cues, from abiotic stress to nodulation, are referred to the following recent articles that review the current knowledge (Oldroyd et al., 2011; O'Brien and Benková, 2013; Suzaki and Kawaguchi, 2014; Zwack and Rashotte, 2015).

HISTORY AND OVERVIEW

Austrian plant physiologist Georg Haberlandt was the first to culture isolated cells of higher plants. Even though he failed to induce divisions in any of these cells, he believed in the universal potential of a single plant cell and was convinced "one could successfully cultivate artificial embryos from vegetative cells" (Haberlandt, 1902). With a slightly different focus, Philip White wanted to study cellular metabolism in a completely undifferentiated tissue where all cells are equal and thus have similar influences on one another. The experimental system he proposed consisted of cultured cells that are "undifferentiated yet capable of unlimited growth" (White, 1939). Almost simultaneously with two French researchers, he published sustained growth of cells derived from root explants (Gautheret, 1939; Nobécourt, 1939). However, all of the initial explants used by these researches included meristematic cells. It was the addition of coconut water to the medium that finally allowed cultivation of recalcitrant tissues, as

was first demonstrated with very early *Datura stramonium* embryos (van Overbeek et al., 1941). The laboratory of Folke Skoog set out to identify the growth-promoting factor from coconut milk using cultivation assays of tobacco stem pieces. Increasing activity was found in coconut meat, yeast extract and eventually autoclaved herring sperm DNA, from which the responsible molecule was isolated. It was given the name kinetin for its cytokinesis promoting activity (Fig. 1a) (Miller et al., 1955, 1956). Most likely it was also a cytokinin in the phloem exudates that stimulated cell division in wounded potato tubers, as had been reported by Haberlandt much earlier (Haberlandt, 1913). With a purified cytokinin at their disposal, researchers set out to test the power of the novel substance, and they identified a number of plant responses, frequently in synergism or antagonism with auxin. Skoog and Miller's famous and still frequently cited report from 1957 (Skoog and Miller, 1957) where they describe how the ratio of cytokinin to auxin determines the organ identity that develops from cultured tissue marked the start. Later, cytokinin's action in counteracting leaf senescence was discovered (Richmond and Lang, 1957), followed by its antagonistic role with auxin in apical dominance (Wickson and Thimann, 1958), and additional examples are continuously

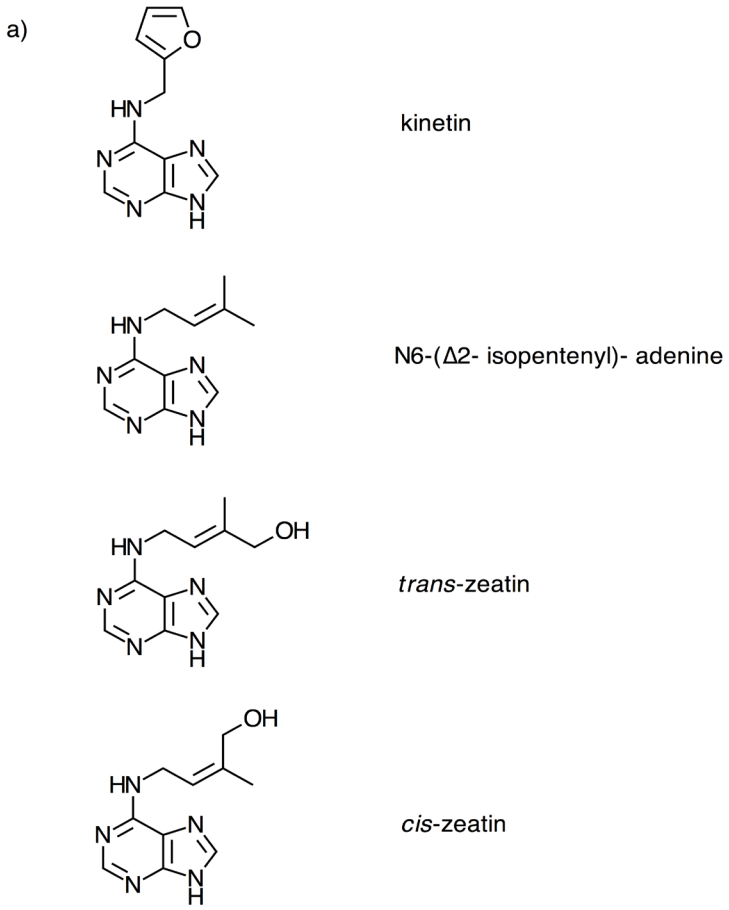

Fig. 1 | Cytokinin structure and biosynthesis

a) Structures of the first isolated cytokinin, kinetin and of prevalent isoprenoid cytokinins occuring in plants. b) Current model of isoprenoid biosynthesis. Plant IPTs preferentially use ADP or ATP over AMP as isoprenoid acceptor creating iP riboside 5'-diphosphate or iP riboside 5'-triphosphate, respectively. Here, only the ADP and resulting diphosphates are shown and downstream processings are only shown for tZ but are valid also for iP and cZ. Light gray arrows indicate reactions which are not well characterized. Glucose units are shown in blue, isoprenoid side chains are shown in brown.

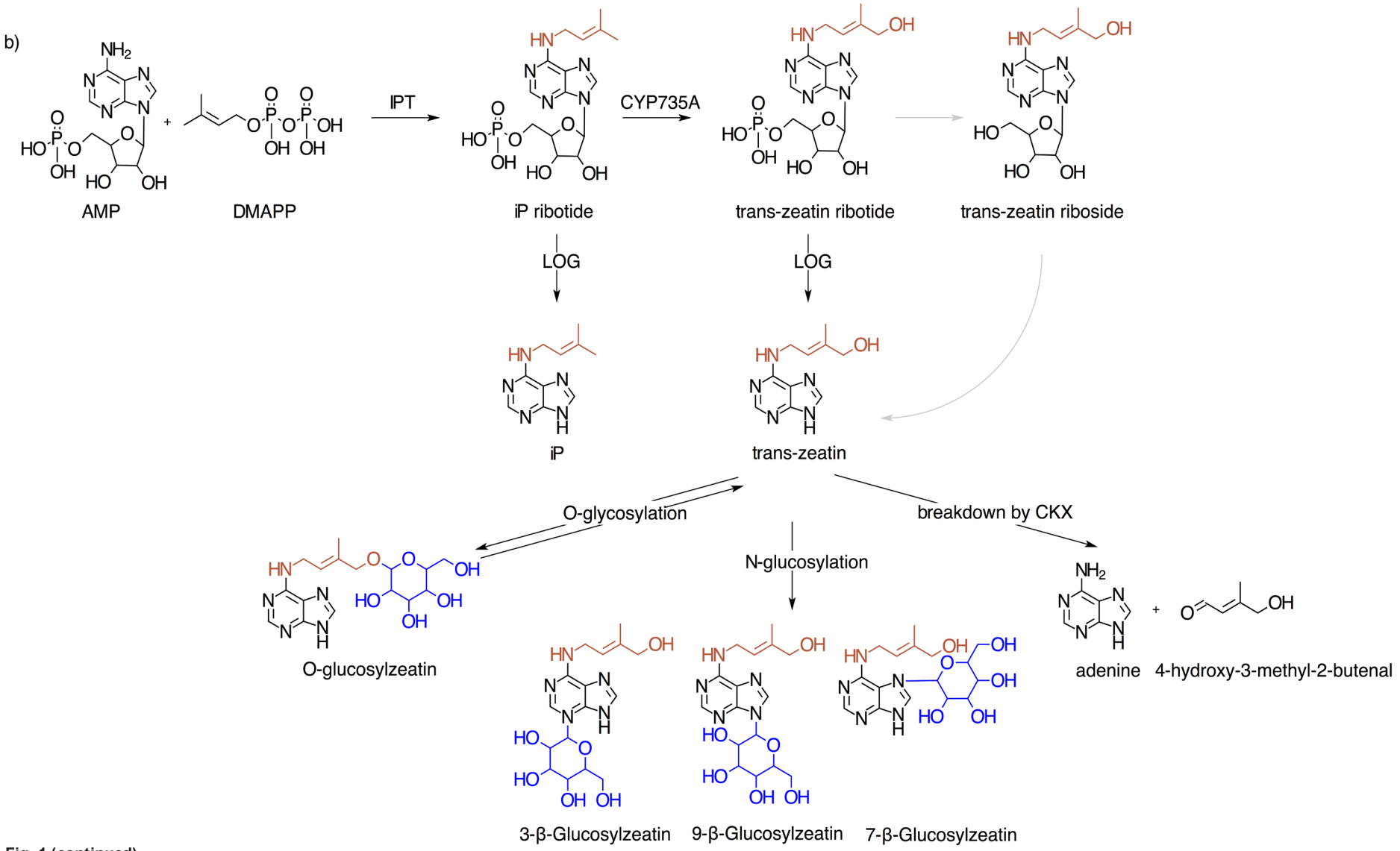

Fig. 1 (continued)

being described (Moubayidin et al., 2009; Bishopp et al., 2011a; O'Brien and Benková, 2013; Schaller et al., 2015). After the first natural cytokinin, zeatin, was isolated from corn endosperm (Miller, 1961), more cytokinins were isolated from natural sources (Fig. 1a) (Mok and Mok, 2001). Parallel efforts were made to test a range of different synthetic molecules to understand the relation between structure and function of active cytokinins (Skoog et al., 1967; Mok et al., 1978). Many enzymes involved in biosynthesis, transport, modification and degradation of cytokinins have been identified and can be considered part of the cytokinin signalling network. These enzymes control the amount of active cytokinins that trigger the response in a given cell (Fig. 1b).

ELUCIDATION OF THE SIGNAL TRANSDUCTION PATHWAY

An activation tagging screen led to the identification of CYTOKININ INDEPENDENT1 (CKI1) that, upon overexpression, induced growth of cultured tissue similar to exogenously added cytokinin. The domains of CKI1, a transmembrane protein with an N-terminal sensing domain, an intracellular histidine (His) kinase, transmitter and receiver domain were implicating that cytokinins activate a multistep two-component signalling system (Kakimoto, 1996). Two-component signalling systems are bacteria's prevalent signalling pathways. Typically, ligand binding triggers His kinase activity causing autophosphorylation on a conserved His residue within the transmitter domain. The activating phosphoryl group is then transferred to a conserved aspartate (Asp) residue in the receiver domain of a response regulator (RR), which induces a cellular response (West and Stock, 2001). Cytokinin signalling realises a multi-step version of phosphorelay, with an additional intermediate step (Fig. 2) (Urao et al., 2000; West and Stock, 2001). The availability of the *Arabidopsis* genome sequence allowed compiling a list of candidate genes with motifs characteristic of a putative role in two-component signalling, including CKI1, ARABIDOPSIS HISTIDINE KINASES (AHK), ARABIDOPSIS HISTIDINE TRANSFERASES (AHP) and ARABIDOPSIS RESPONSE REGULATORS (ARR) with the latter being classified into type-*A*, type-*B* and type-*C* (Fig. 2) (Imamura et al., 1999; Suzuki et al., 2000). It turned out that CKI1 does not bind cytokinin, and the role of its conserved extracellular sensing domain is still unclear (Hejátko et al., 2009). The first true cytokinin receptor, AHK4/CRE1/WOL was independently discovered by three groups (Mähönen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi, 2001). Also *AHK2* and *AHK3* were later identified as cytokinin receptors (Hwang and Sheen, 2001). The AHP proteins connect the receptors with the nuclear RRs by shuttling between cytoplasm and nucleus to transfer the activating phosphoryl group to receiver domains of RRs (Hwang and Sheen, 2001; Punwani et al., 2010). Diligent work using combinations of biochemical, physiological and genetic approaches performed by several research groups confirmed the core cytokinin signalling cascade as described above (Hwang and Sheen, 2001; Imamura et al., 2001; Yamada et al., 2001; Suzuki et al., 2002; Higuchi et al., 2004; To et al., 2004). As each step of the signalling cascade is supported by families with multiple members, higher order mutants of each family were required

to obtain visible phenotypes, which were generated for all of the relevant signalling components (To et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Riefler et al., 2006; Yokoyama et al., 2007; Deng et al., 2010).

FROM CYTOKININ PRODUCTION TO SIGNALLING RESPONSE

CYTOKININ IN PLANTS

Cytokinins occur in plants as free bases, nucleosides (ribosides), glycosides (*O*- and *N*-glycosides) and nucleotides (Fig. 1b). The free bases represent the active forms, while the ribosides have low activities (Yamada et al., 2001; Lomin et al., 2015). The occurrence, distribution and variation of individual cytokinins depend on plant species, tissue, and developmental stage (Sakakibara, 2006). Interestingly, the inactive cytokinins are much more abundant compared to the free bases (Takei et al., 2004b; Miyawaki et al., 2006; Svačinová et al., 2012; Kiba et al., 2013), which indicates that the concentration of active cytokinins is tightly controlled to prevent unregulated signalling. This is achieved by coordination of the enzymes involved in biosynthesis, modification and degradation of cytokinins.

Besides kinetin, other structurally related compounds with cytokinin activity have been identified. While synthetic cytokinins can be structurally diverse, naturally occurring cytokinins are all comprised of adenine derivatives but differ in the side chain attached to the N^6-position of the purine (Mok and Mok, 2001). Two classes of side chains can be distinguished; the isoprenoid cytokinins and the aromatic cytokinins (Strnad, 1997; Mok and Mok, 2001). Little is known about the biosynthesis of the latter, and they have been identified in only a limited number of plant species so far. The best-studied isoprenoid cytokinins are N^6-(Δ^2-isopentenyl)-adenine (iP), zeatin, and dihydrozeatin. Zeatin's side chain contains a double bond and its hydroxy group can therefore be oriented in a *trans*- or *cis*- configuration representing *trans*-zeatin (tZ) or *cis*-zeatin (cZ), respectively. The side chain of dihydrozeatin is saturated, while the functional group of the iP side chain is a methyl compared to a hydroxymethyl in zeatin (Fig 1a). In *Arabidopsis*, tZ and iP are the prevalent cytokinins, while in maize, chickpea and rice, cZ is common as well (Mok and Mok, 2001; Sakakibara, 2006). In order to determine the structural requirements for cytokinin activity and because of the strong agricultural interest in cytokinins' potential to increase growth and resistance to abiotic stress, several chemicals were synthesized and assayed for activity. The most commonly known synthesized cytokinins are benzyladenine (BA), an aromatic cytokinin that was later found to occur naturally in some plant species, and thidiazuron, which has been used as a defoliant in cotton fields (Mok et al., 1982). Thidiazuron falls into the group of urea-type cytokinins.

CYTOKININ SYNTHESIS

The first dedicated step in the biosynthesis of isoprenoid cytokinins is the addition of a prenyl group of dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP) onto AMP, ADP or ATP yielding iP ribotides. This reaction is catalysed by ISOPENTENYLTRANSFERASES (IPTs) (**Fig. 1b**) (Kakimoto, 2001; Takei et al., 2001a). The *Arabidopsis* genome encodes nine IPTs of which seven, IPT1, IPT3-8 preferably use ATP and ADP as isoprenoid acceptors (Kakimoto, 2001; Takei et al., 2001a). IPT2 and 4 localize to the cytoplasm, IPT7 to mitochondria, and IPT1, 3, 5 and 8 to plastids (Kasahara et al., 2004). IPT2 and IPT9 catalyse the isopentylation of tRNA, which provides a source for cZ-type cytokinins (Miyawaki et al., 2006). Initially, no visible phenotypes were detected for the *atipt2,9* double mutant, which indicated that tRNA breakdown as cytokinin source is negligible (Miyawaki et al., 2006). Upon closer inspection, a shortening of the main root, as well as a decreased number of lateral roots were described. This effect is due to a smaller meristematic region in the root procambium leading to additional protoxylem files, suggesting that cZ-type cytokinins control protoxylem differentiation (Köllmer et al., 2014). The quadruple mutant *ipt1,3,5,7* is retarded in growth and shows decreased levels of iP, tZ and derivatives.

The iP ribotide produced by IPT action can be converted into the tZ ribotide by cytochrome P450 monooxygenases CYP735A (**Fig. 1b**) (Takei et al., 2004b; Kiba et al., 2013). A two-step reaction that converts the ribotides to the ribosides with subsequent cleavage of the sugar moiety was expected for the production of the free bases, and enzymes catalysing the first step of the reaction have been reported (Kopečná et al., 2013), but their role *in planta* is not yet known. Instead, the free bases are directly released from the cytokinin ribotides by the LONELY GUY (LOG) protein family (**Fig. 1b**) (Kurakawa et al., 2007; Kuroha et al., 2009; Tokunaga et al., 2012). Based on cytokinin measurements and mutant phenotypes, the direct pathway via LOG represents the main route of activation. LOG members localize to the cytoplasm or nucleus and are expressed in specific domains throughout the plant. Similar to the *IPTs*, the *LOG* expression domains show relatively little overlap with the cytokinin response domains, suggesting transport of cytokinins within tissues (**Fig. 3**) (Kurakawa et al., 2007; Kuroha et al., 2009; Chickarmane et al., 2012; Tokunaga et al., 2012). The notable exception is the provasculature of the embryo, where *LOG* expression and cytokinin response largely coincide (Zürcher et al., 2013; De Rybel et al., 2014). In contrast to early notions that cytokinins are produced in roots only, it is now clear that they are synthesised throughout the plant. IPTs are expressed in several cell types and organs including virtually all aerial organs (Miyawaki et al., 2004; Kuroha et al., 2009). However, the CYP735 proteins, which convert iP ribotides into tZ ribotides, predominantly localize to root tissue (Takei et al., 2004b). Hence, tZ-type cytokinins are more readily produced in roots but are transported to other organs.

CYTOKININ MODIFICATION AND DEGRADATION

Modified cytokinins constitute pools that can rapidly be activated when needed, and thus contribute to control the levels of active cytokinins, the free bases (Kiran et al., 2012). Ribosides and ribotides are therefore thought to serve as storage or transport forms. Indeed, the largely inactive iP- and tZ-ribosides were found to be the major long-range transport forms in plants (Beveridge et al., 1997; Takei et al., 2001b; Corbesier et al., 2003; Kudo et al., 2010).

In *O*-glycosylation, glucosyl- or xylosyl-transferases attach a sugar moiety to the oxygen in the side chain of zeatin or dihydrozeatin (**Fig. 1b**). *O*-glycosylated cytokinins are not active in bioassays but can be re-activated to free bases by beta-glucosidases (Brzobohatý et al., 1993). Overexpression of the main *O*-glycosidase, UGT85A1 increases the pool of *O*-glycosylated cytokinins and decreases the sensitivity to exogenously applied tZ. *N*-glycosylation targets the purine ring and appears to be irreversible and therefore represents a mechanism for definitive scavenging of unwanted cytokinins (Parker and Letham, 1973). Consistent with this view, overexpression of *N*-glycosylation enzymes, AtUGT76C1 and AtUGT76C2, increases the pool of *N*-glycosylated cytokinins and reduces the sensitivity to exogenously added tZ. The opposite effects are observed in the corresponding mutants. Interestingly, neither *UGT85A1* nor *UGT76C* overexpressing plants show apparent phenotypes and the level of tZ remains constant, most likely because plants compensate and adapt to the challenges (Wang et al., 2011; Jin et al., 2013; Wang et al., 2013).

Levels of cytokinins with an unsaturated side chain, tZ, cZ and iP, their ribosides and ribotides, are further regulated via the irreversible oxidative cleavage of the N^6-side chain by CYTOKININ DEHYDROGENASE/OXIDASES (CKXs) (**Fig. 1b**) (Schmülling et al., 2003; Galuszka et al., 2007). *CKXs* have first been cloned from *Zea mays* (Houba-Hérin et al., 1999; Morris et al., 1999) but were then found in many species including rice, *Arabidopsis*, and the plant pathogen *Rhodococcus fascians* (Crespi et al., 1992; Werner et al., 2001; Schmülling et al., 2003). In *Arabidopsis*, there are 7 *CKX* family members *CKX1-CKX7*, whose gene products localize to the vacuole, the extracellular space, or, with CKX7 representing the only member there, to the cytosol (Werner et al., 2003; Köllmer et al., 2014). Because CKXs operate on unsaturated side chains, DHZ and aromatic cytokinins are resistant to CKX activity. Also glycosylation protects cytokinins from oxidative cleavage (Werner et al., 2006). Overexpression of *CKXs* reduces the amount of active cytokinins and causes strong phenotypes (Werner et al., 2003). Reduced expression of *OsCKX2* in rice increases cytokinin levels and concomitantly grain production (Ashikari et al., 2005). In *Arabidopsis*, mutations in single *CKX* genes show no obvious phenotypes, however a *ckx3ckx5* double mutant displays a number of phenotypes consistent with increased cytokinin activity (Bartrina et al., 2011). The expression patterns of *CKXs* are specifically regulated suggesting that CKXs are central regulators controlling the pools of active cytokinins (Werner et al., 2006).

CYTOKININ PERCEPTION PATHWAY

The members of the ARABIDOPSIS HISTIDINE KINASE (AHK) family are transmembrane proteins with (His) kinase activity (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi, 2001; Nishimura et al., 2004). Binding of a cytokinin to the AHK is mediated via the CHASE (cyclases/histidine kinases associated sensory extracellular) domain, which is thought to elicit a conformational change in the receptors (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001; Hothorn et al., 2011). The AHKs are present at the membrane as dimers and supposedly transphosphorylate each other on a conserved His residue in the transmitter domain upon binding (West and Stock, 2001). The phosphoryl group is transferred onto a conserved Asp residue on the receiver domain within the receptor itself. AHK4 but not AHK2, nor AHK3, harbours intrinsic phosphatase activity at a constant level. Below a certain threshold of ligands, phosphatase activity dominates over the His kinase activity, which results in removal of activating phosphates. This seems to buffer the system against spurious activation (Mähönen et al., 2006b).

A mutation in *AHK4* underlies the *wooden leg* (*wol*) mutation, which displays aberrant root vasculature consisting of protoxylem only (Scheres et al., 1995; Mähönen et al., 2000; Suzuki et al., 2001). The *Arabidopsis* triple mutant *ahk2ahk3ahk4* is insensitive to cytokinin in various assays and shows reduced meristematic activity causing dwarfed growth. Crossings of such triple mutants to a wild-type plant were unsuccessful indicating that male and female gametogenesis depend on receptor function (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Growth and sensitivity assays with calli and seedlings of the different double mutant combinations revealed cytokinin perception in roots mainly depends on *AHK4* function while *AHK2* and *AHK3* act redundantly predominantly in the shoot (Higuchi et al., 2004; Nishimura et al., 2004). Additionally, *AHK3* is a key player in regulating the onset of senescence (Kim et al., 2006).

From the receiver Asp in AHKs, the phosphoryl group is transferred onto a His in ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER (AHP) proteins. AHPs shuttle between the cytosol and the nucleus to connect between receptors and the nuclear ARABIDOPSIS RESPONSE REGULATOR (ARR) (**Fig. 2**) (Hwang and Sheen, 2001; Punwani et al., 2010). The AHPs transfer the phosphoryl onto ARR members via direct interaction (Hwang et al., 2012; Verma et al., 2015). AHP6 differs from AHP1-5 as it cannot accept an activating phosphoryl group; instead of the conserved Asp, it carries an inert asparagine (Asn) at the relevant position and therefore does not participate in the phosphorelay (Hutchison et al., 2006; Mähönen et al., 2006a). AHP6 is however important to attenuate signalling. AHP6 competes with AHP1-5 for access to the receptors, decreasing productive phosphorelay (**Fig. 2**). This contributes to sharpen the signalling output domains e.g. in the embryo, the vasculature and the root apical meristem (RAM) (Mähönen et al., 2006a; Bishopp et al., 2011b; Besnard et al., 2014; Ohashi-Ito et al., 2014).

The *ahp1,2,3* triple mutant is less sensitive to cytokinin as are the higher order mutants (Hutchison et al., 2006). Alike the *wol* mutant, the quintuple mutant *ahp1,2,3,4,5* fails to develop phloem and metaxylem vessels (Mähönen et al., 2000; Hutchison et al., 2006; Deng et al., 2010).

The *Arabidopsis* genome encodes 23 ARRs (Sakai et al., 1998; Suzuki et al., 2002; Mason et al., 2004; To et al., 2004; Hutchison et al., 2006; Argyros et al., 2008). The family of *ARRs* is classified into the groups of *A-, B-* and *C*-type ARRs according to C-terminal differences (Imamura et al., 1999; D'Agostino et al., 2000; Müller, 2011). Type-*B* ARRs have a Myb-transcription factor domain for DNA-binding and act as transcriptional activators (Kiba et al., 1999; Mason et al., 2004).

Type-*A* ARRs lack DNA binding and transactivation domains and attenuate signalling

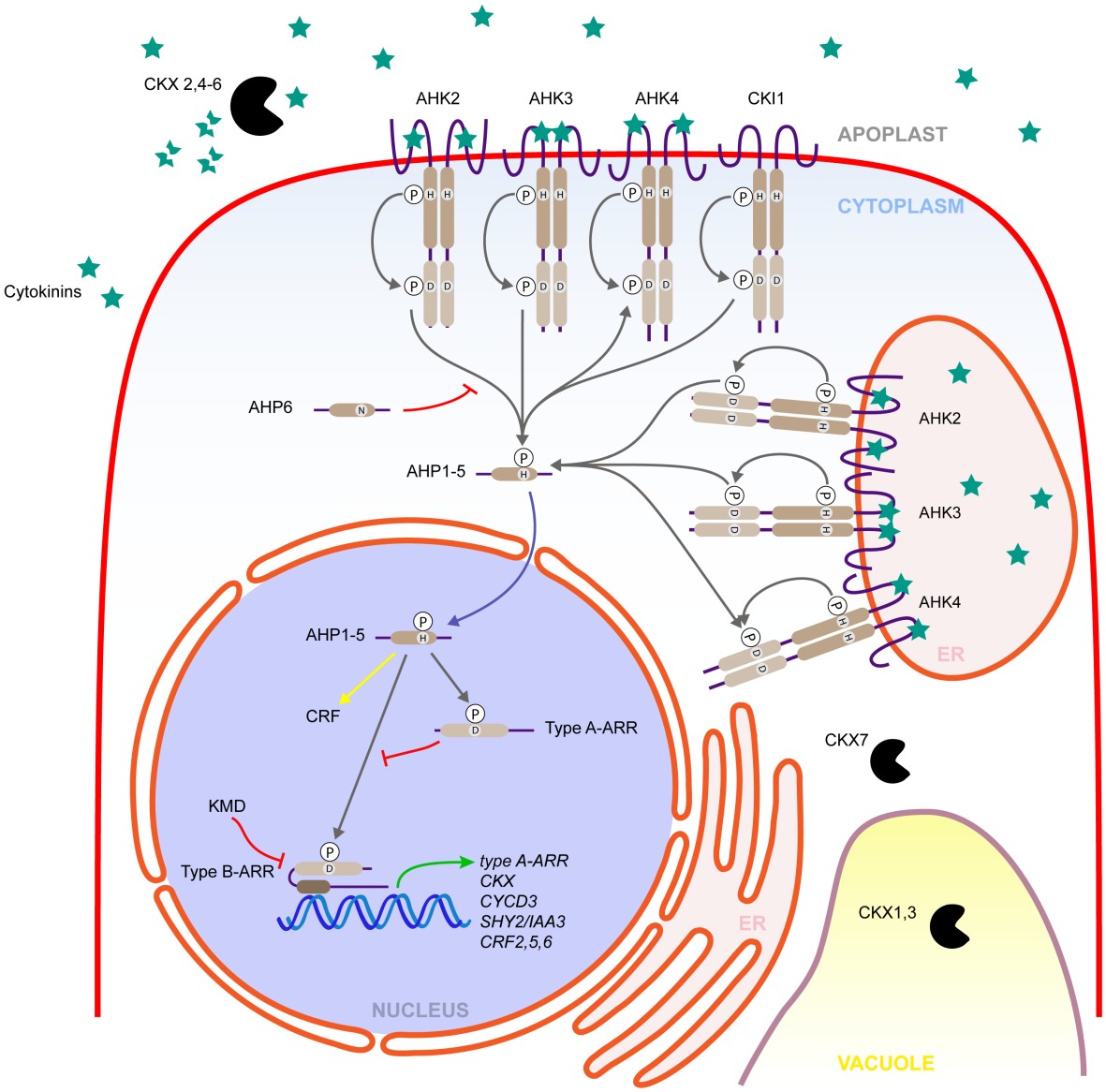

Fig. 2 | Current model of the cytokinin signaling pathway.
Cytokinins are perceived by AHK receptors. Phosphorelay transmits the phosphoryl group via AHPs onto the nuclear ARRs. CKI1 can elicit phosphorelay independent of cytokinin. CKXs in the cytosol and in the apoplast regulate cytokinin levels by cleavage. Gray arrows denote phosphorelay transfer; blue arrows indicate protein movement; yellow arrows indicate posttranscriptional activation; red arrows indicate posttranscriptional inhibition; green arrows indicate transcriptional activation. AHK, ARABIDOPSIS HISTIDINE KINASE; CKI1, CYTOKININ INDEPENDENT1; AHP, ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN; ARR, ARABIDOPSIS RESPONSE REGULATOR; CRF, CYTOKININ RESPONSE FACTOR; KMD, KISS ME DEADLY; CKX, CYTOKININ OXIDASE.

(Brandstatter and Kieber, 1998; D'Agostino et al., 2000; Rashotte et al., 2003). Type-*A ARRs* are among the type-*B* ARR target genes, which establishes a negative feedback loop (Fig. 2) (Brandstatter and Kieber, 1998; D'Agostino et al., 2000; Hwang and Sheen, 2001; Rashotte et al., 2003; To et al., 2004, 2007).

The expression of type-*A ARR*, in particular *ARR5* or *ARR6*, has been used to monitor transcriptional activity in response to cytokinin signalling (D'Agostino et al., 2000; Hwang and Sheen, 2001; Nisler et al., 2010; Chang et al., 2013). However, transcription of type-*A ARRs* is not exclusively controlled by cytokinin but depends on secondary signals and tissue-specific factors, which limits their use as reporters. The synthetic promoters *TCS* and *TCSn* (Two Component signalling Sensor new) were constructed to overcome these shortcomings (Müller and Sheen, 2008; Zürcher et al., 2013). These synthetic promoters harbour the concatemerised DNA binding motifs for the type-*B* RRs in optimized arrangement for specific, sensitive and tissue-independent transcriptional activation in response to cytokinin signalling (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). *TCS*-based reporters have been instrumental in precisely reporting cytokinin signalling, leading to refined models of cytokinin activities in numerous contexts (Leibfried et al., 2005; Gordon et al., 2009; Zhao et al., 2010; Bielach et al., 2012; Chickarmane et al., 2012; Zúñiga-Mayo et al., 2014) and uncovering novel cytokinin functions in the embryo, gynoecium and stem cell niche of the leaf axils (Müller and Sheen, 2008; Bencivenga et al., 2012; Marsch-Martínez et al., 2012; Wang et al., 2014).

Multiple knockouts of type-*B ARRs* lead to decreased cytokinin sensitivity and inherently have a decreased cytokinin response. Triple mutants of *arr1,10,12* are dwarfed with short main roots deficient of metaxylem (Mason et al., 2005; Argyros et al., 2008; Ishida et al., 2008). In contrast, higher order mutants in the type-*A ARRs* such as the *arr3,4,5,6* quadruple mutant show enhanced cytokinin sensitivity in various assays and display elongated petioles (To et al., 2004).

Compared to *A*- and *B*-type ARRs, the role of the *C*-type ARRs is less well charcterized. Their expression is restricted to reproductive tissue and is not affected by cytokinin signalling. Nevertheless, their ectopic expression strongly affects cytokinin signalling output (Kiba et al., 2004; Gattolin et al., 2006), therefore they might serve as modulators of cytokinin signalling under yet unknown conditions. Incidentally, the *C*-type *ARR, ARR22*, was found to be upregulated in the chalaza by wounding of the developing seed (Gattolin et al., 2006; Horák et al., 2008). However, mutants show no obvious phenotypes under standard growth conditions.

Additional factors have been found to modulate the core components, or to offer alternative routes of signalling. The type-*A* ARR proteins are stabilized by cytokinin, which affects their efficiency and helps control the duration of a stimulus (Ren et al., 2009; Kim et al., 2012). In the absence of such stabilization, type-*A* ARR levels are regulated by proteolysis. Intriguingly, the type-*B* ARR, ARR2 is targeted for proteasomal degradation upon cytokinin signalling (Kim et al., 2012).The KISS ME DEADLY (KMD) family of F-Box proteins interacts directly with the *B*-type ARRs to target them for

degradation via the proteasome (Fig. 2) (Kim et al., 2013a, b). The CYTOKININ RESPONSE FACTORs (CRFs) act in parallel to the *B*-type ARRs. CRFs are members of the large AP2 family of transcription factors and their expression is induced by cytokinin (Fig. 2). Many of the genes induced by CRFs are shared with the targets of type-*B* ARRs (Rashotte et al., 2006). Less is known about the functional relevance of the *CRFs*, but a recent report attributes a role to *CRF6* in negative regulation of leaf senescence (Zwack et al., 2013). Furthermore, yeast-two-hybrid analyses have shown that CRFs can directly interact with the AHPs (Cutcliffe et al., 2011).

LIGAND MEETS RECEPTOR: CYTOKININ TRANSPORT

Reciprocal grafting experiments between *ipt1,3,5,7* mutant and wild-type plants indicated that tZ-type cytokinins are transported shootwards in xylem, while iP-type cytokinins move preferentially from the shoot to the root via symplastic connections in the phloem (Takei et al., 2001b; Corbesier et al., 2003; Matsumoto-Kitano et al., 2008; Shimizu-Sato et al., 2009; Kudo et al., 2010; Bishopp et al., 2011c). Root-to-shoot transport of tZ was further demonstrated by analysing the *CYP735* double mutants (Kiba et al., 2013). Interestingly, the mutants showed defects mainly in the shoot, demonstrating that root-borne tZ-type cytokinins control shoot growth. The AtABCG14 transport protein has recently been found to be implicated in xylem-loading of mainly tZ-type cytokinins in roots (Ko et al., 2014; Zhang et al., 2014). *AtABCG14* is expressed primarily in the root vasculature and the loss of *AtABCG14* expression significantly reduces the amount of tZ-type cytokinins in the xylem (Ko et al., 2014). Cytokinins instead seem to accumulate in the root as seen by enhanced root cytokinin signalling activity (Zhang et al., 2014).

In many contexts, cytokinin production occurs in different cells than cytokinin perception, suggesting that cytokinins are transported from the producing cells to the receiving cells and act in a paracrine way. Specifically, in many tissues the expression of *IPT* genes does not overlap with cytokinin signalling activities (Zürcher et al., 2013), and the same is true for *LOG* or *CYP* genes (Fig. 3). For example during lateral root formation, IPT5 and LOG4 levels are high in the primordial founder cells of the pericycle where the cytokinin response is turned off (Miyawaki et al., 2004; Zürcher et al., 2013; Chang et al., 2015). Similarly, in the shoot apical meristem (SAM), LOG4 locates to the L1 layer, while cytokinin output is found in the underlying cell layers of the organizing centre (Fig. 3) (Chickarmane et al., 2012; Zürcher et al., 2013).

Furthermore, cytokinin-binding proteins important for biosynthesis, modification or signalling are found in different cellular compartments, including apoplast, cytosol, chloroplast and the endoplasmic reticulum, which implies that cytokinins are transported across membranes.

The cytokinin receptors AHK2, AHK3 and AHK4 were reported to localize to ER membranes in addition to their localization to the plasmalemma (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011) with their sensing domains protruding into the lumen of the ER. However, the functional relevance of ER-localized receptors

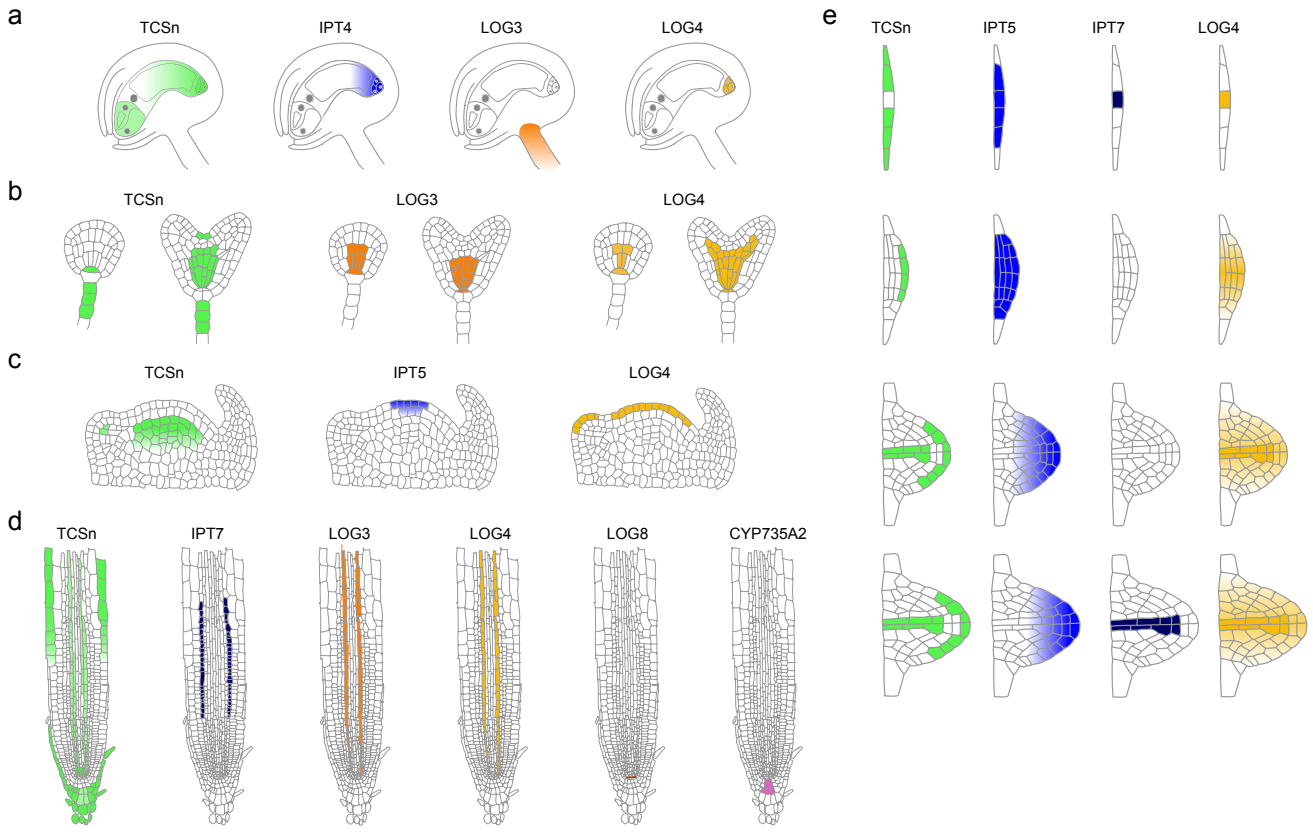

Fig. 3 | Comparison of cytokinin signalling and expression patterns of relevant cytokinin synthesis genes. Expression patterns of *IPT*, *LOG* and *CYP*, and *TCSn* are shown during (a) female gametogenesis, (b) during embryogenesis, (c) in the SAM, (d) in the RAM, (e) and during different stages of lateral root emergence.

versus plasma membrane localized receptors has not been shown yet. The potential ER localization of the receptors and the different subcellular localizations of the enzymes involved in cytokinin metabolism are indicative of active transport of cytokinin species, as no free diffusion has been observed (Cedzich et al., 2008). The family of EQUILIBRATIVE NUCLEOSIDE TRANSPORTERs (ENTs) was shown to transport cytokinin ribosides in yeast heterologous assays (Hirose et al., 2005, 2008). In rice, the *ENT* members are expressed in the vasculature suggesting that they mediate uptake from transported cytokinins (Hirose et al., 2005).

As for short-range, or intracellular transport of cytokinins, another family of transporters has been proposed; the PURINE PERMEASEs (PUPs) (Gillissen et al., 2000; Bürkle et al., 2003; Cedzich et al., 2008). Individual PUP family members were shown to be able to transport adenine and a variety of adenine derivatives such as caffeine and different species of cytokinins into yeast and *Arabidopsis* cell cultures (Bürkle et al., 2003). While these members are suggested to localize to the plasma membrane mediating uptake, other members of the PUP family might reside in endomembranes to allow transport between organelles. The *Arabidopsis* genome encodes more than 20 of these PUPs but further studies are needed to address their function in cytokinin transport.

INTEGRATION OF CROSSTALK

Cytokinins exert many different functions during the course of a plant's life. In most instances, cytokinin function is modulated, supported or antagonized by other hormones and/or environmental stimuli. Crosstalk occurs at many levels ranging from cytokinin biosynthesis, perception, signalling output, or cytokinin modification, transport and degradation.

AUXIN AND OTHER HORMONES

Auxin is the most prominent signal known to interact with cytokinin (Zhao, 2008; Bishopp et al., 2011a; El-Showk et al., 2013; O'Brien and Benková, 2013; Schaller et al., 2015). In many cases, auxin's effect on cytokinin is antagonistic, as is nicely illustrated in the crosstalk of these two hormones during tissue regeneration (Skoog and Miller, 1957), root stem-cell specification (Müller and Sheen, 2008), or lateral root formation (Benková et al., 2003; Werner et al., 2003; Marhavý et al., 2013, 2014). On the other hand, auxin and cytokinin have also been found to act synergistically e.g. in the SAM (Leibfried et al., 2005; Zhao et al., 2010). Also, the hormones gibberellic acid, ethylene and abscisic acid have been shown to modulate cytokinin signalling in specific contexts (Moubayidin et al., 2010; Guan et al., 2014; Lu et al., 2014; Pacifici et al., 2015; Žďárská et al., 2015). Some of these processes and the roles cytokinin takes over therein are described later in more detail.

LIGHT

Besides using light as energy source, plants are influenced by light quality and intensity in multiple ways. Cytokinin signalling mediates and modulates a number of light-dependent processes, and its involvement has been elucidated at the molecular level. The *A*-type ARR4, was shown to interact with the phytochrome receptor PhyB. ARR4 recognizes and binds both, the active Pfr and inactive Pr form of PhyB, but stabilizes the active Pfr form thereby affecting red-light signalling (Sweere et al., 2001). In turn, a high level of far-red light, as found in canopy shade induces growth arrest of leaf primordia via auxin-dependent *CKX6* induction (Carabelli et al., 2007). Wild-type seedlings grown in the dark display an etiolated morphology; they grow a long hypocotyl in an effort to reach light, do not expand cotyledons or leaves and are pale due to the lack of chlorophyll. Addition of high doses of cytokinin to dark-grown seedlings induces their de-etiolation, indicating that etiolation depends on low cytokinin levels (Chory et al., 1994). Additionally, the *elongated hypocotyl hy5* mutant, which displays skotomorphogenesis when grown in light, is irresponsive to exogenous cytokinin in root inhibition and callus induction assays (Cluis et al., 2004). *HY5* encodes a bZIP transcription factor that promotes photomorphogenic development. In the dark, it is negatively regulated by the ubiquitin ligase COP1 (Osterlund et al., 2000). It

was found that cytokinin stabilizes the HY5 protein and protects it from degradation (Vandenbussche et al., 2007). Furthermore, plants with compromised cytokinin signalling activity such as the *ahk2ahk3* double mutant exhibit increased photoinhibition after high-light treatment indicating a function of cytokinin in light stress response (Cortleven et al., 2014).

NITRATE AND NITRIC OXIDE

Cytokinin levels are positively correlated with the amount of nitrate or ammonium in the medium or in the soil. This can be in part explained by *IPT5* whose expression in the root is similarly correlated. In the shoot, however, the expression levels of *IPT5* decrease with increasing levels of soil or medium nitrate (Takei et al., 2004a). This represents a long-term adaptation mechanism through which the plant signals nutritional information from the root to adjust growth. On the other hand, nitrogen-starved *Arabidopsis* seedlings induce expression of *IPT3* upon NO_3^- resupply, which illustrates short-term, rapid adaptation to changes in a nutritional status (Takei et al., 2004a). This observation might underlie the finding of increased cytokinin levels in maize roots, xylem and leaves after nitrogen supplementation (Takei et al., 2001b).
Nitric oxide (NO) was shown to directly interact with zeatin *in vivo* creating nitrated cytokinin species and thereby lowering endogenous NO levels (Liu et al., 2013). Conversely, NO regulates cytokinin signalling by inhibiting phosphorylation of AHP1 through S-nitrosylation (Feng et al., 2013). This may allow the plant to adjust growth rates in accordance with the perceived redox conditions.

CYTOKININ FUNCTIONS THROUGHOUT DEVELOPMENT

Mutations that alter cytokinin signalling often cause growth retardation (Werner et al., 2003; Nishimura et al., 2004; Miyawaki et al., 2006; Kuroha et al., 2009; Tokunaga et al., 2012), alterations in root architecture (Riefler et al., 2006; Zhang et al., 2011; Chang et al., 2013; Köllmer et al., 2014), defects in root vasculature development (Scheres et al., 1995; Mähönen et al., 2000; Hutchison et al., 2006; Ishida et al., 2008), changes in apical dominance and branching patterns (Sachs and Thimann, 1967; Giulini et al., 2004), sterility or infertility (Higuchi et al., 2004; Nishimura et al., 2004; Cheng et al., 2013), embryo or seedling defects (Müller and Sheen, 2008; Deng et al., 2010), or changes in seed size (Miyawaki et al., 2006; Riefler et al., 2006; Argyros et al., 2008; Ishida et al., 2008; Bartrina et al., 2011; Tokunaga et al., 2012). Furthermore, changes in cytokinin signalling or cytokinin sensitivity may affect stress tolerance (Laffont et al., 2015), onset of senescence (Gan and Amasino, 1995; Kim et al., 2006), or nodulation in legumes (Sasaki et al., 2014). Many of these effects observed in plants with altered cytokinin signalling are caused by changes in meristematic activities reflecting the importance of balancing cell division and differentiation.

MALE AND FEMALE GAMETOPHYTE DEVELOPMENT

Gametogenesis in angiosperms proceeds in the protected surroundings provided by the flower. The female gametophyte (FG) develops within the ovule, the male gametophyte (MG) within the anther primordia. Connections between FG development and phosphorelay signalling were postulated after it was found that *cki1* mutant plants displayed FG lethality (Pischke et al., 2002; Hejátko et al., 2003). Later, seeds could be obtained at a low frequency from a different allele but these were enlarged compared to wild-type seeds (Deng et al., 2010). CKI1 can induce a cytokinin response via the AHPs independently of *AHK* function. Consistently, an *ahp1,2,3,4,5* quintuple mutant often showed defects in FG development similar to these observed in the *cki1* mutant (Deng et al., 2010). In addition, cytokinin-dependent AHP activation is required, as triple *ahk2,3,4* mutants also show defects in ovule development (Kinoshita-Tsujimura and Kakimoto, 2011; Cheng et al., 2013). The functionality of the MG also depends on at least one functional receptor, as in triple receptor mutants anthers fail to dehisce and pollen do not maturate properly (Kinoshita-Tsujimura and Kakimoto, 2011). In the FG, cytokinin activity is distributed asymmetrically and shows an increase in the chalazal end, supported by increased expression of *AHK4* and *IPT1* in this locale (Bencivenga et al., 2012; Cheng et al., 2013). It was suggested that cytokinin affects ovule development at least in part by deregulation of *PIN1* as exogenous addition of cytokinin causes ectopic *PIN1* expression, and formation of ectopic ovule primordia (Bencivenga et al., 2012; Ceccato et al., 2013). An increased ovule number has also been observed in the *ckx5ckx6* mutant which is compromised in cytokinin degradation (Bartrina et al., 2011). Application of exogenous cytokinin furthermore results in defects in the patterning of the gynoecium, which in severe cases leads to valveless siliques (Zúñiga-Mayo et al., 2014). While the defects caused by altered phosphorelay signalling indicate a function for cytokinin in gametophyte development, the underlying mechanisms are not completely elucidated and further research will hopefully shed light onto these processes.

EMBRYOGENESIS

During embryogenesis, a single-celled zygote develops into a mature seed. The plant's blueprint with apical-basal and radial axes, and with the major organs and cell types are established during this process (Jürgens, 2001). At the apex of the embryo, centred between the two prospective cotyledons, the SAM is formed while the RAM is established at the opposite end. These stem cell systems are active throughout the sporophyte and control the adult architecture of the plant by building the appropriate organs when needed. The radial organisation includes the different organs of the vasculature. The critical role of cytokinin was already anticipated based on tissue culture experiments (Skoog and Miller, 1957), where cytokinin was both promoting shoot fate and growth, and inhibiting root formation. Recent research has revealed that cytokinin is crucially involved in setting up virtually any aspects of the embryo

bauplan.
A cytokinin response was detected in the prospective shoot meristem and in the provasculature from heart-stage onward (Zürcher et al., 2013). Recent studies have assessed the expression pattern of *LOG* genes and found that *LOG3* and *LOG4* are similarly expressed in the provasculature, which might account for the pattern of cytokinin activity (De Rybel et al., 2014). Intriguingly, *LOG3* and *LOG4* are locally induced by auxin via the transcription factor dimer TARGET OF MONOPTEROS5/ LONESOME HIGHWAY (TMO5/LHW) which creates an intimate interaction between the two phytohormones (De Rybel et al., 2013, 2014; Ohashi-Ito et al., 2014). During earlier development of the embryo, cytokinin signalling is detected in the hypophysis, the founder cell of the embryo root meristem. After its first division, the cytokinin response is maintained in the apical, lens-shaped cell, while signalling is suppressed in the lower, basal cell (Müller and Sheen, 2008). This suppression was found to depend on auxin signalling that directly induces expression of the type-*A ARR7* and *ARR15* (Müller and Sheen, 2008). Ectopic cytokinin signalling caused by an inducible *arr7arr15* double mutant leads to patterning defects (Müller and Sheen, 2008). Interestingly, a stable *arr7arr15* double mutant has milder phenotypes (Zhang et al., 2011) compared to the inducible mutant, suggesting that embryos can specifically compensate the permanent loss of *ARR7* and *ARR15*.

VASCULAR DEVELOPMENT

The plant vasculature mediates transport of photosynthates from source to sink, and water and nutrients from the root to aerial tissues. The *Arabidopsis* vasculature runs as a cylinder of cell files through the plant that is constituted of two halves of procambial tissue intersected by xylem cells with xylem pole cells at their ends. Two files of phloem strands run at the ridge of the procambial cells. Pericycle cells surround the cylinder. The vascular primordium originates from the procambium, which can be traced back to the innermost cells of the globular stage embryo. Through periclinal divisions, the provasculature increases its diameter.
In accordance with their important functions, mutations causing perturbations in cytokinin or auxin signalling often cause severe defects in vascular tissue formation (Scheres et al., 1995; Rouse et al., 1998; Miyawaki et al., 2006; Mähönen et al., 2006a, b; Yokoyama et al., 2007; Hejátko et al., 2009; Bishopp et al., 2011b; Miyashima et al., 2013; Köllmer et al., 2014). The *wol* mutation in the *AHK4* receptor revealed a role for cytokinin during embryonic vascular development (Mähönen et al., 2000, 2006a, b; Bishopp et al., 2011b). *wol* mutants show a reduced vascular system without metaxylem and phloem, but with protoxylem (Scheres et al., 1995). Similar phenotypes can be observed with multiple *ahk*, *ahp* and type-*B arr* mutants (Nishimura et al., 2004; Hutchison et al., 2006; Argyros et al., 2008). Modelling of embryonic vascular tissue formation has shown that auxin defines areas of cytokinin synthesis, while the produced cytokinin non-cell autonomously limits levels of active auxin efflux carrier

PIN1 at the cell membrane (De Rybel et al., 2014; Marhavý et al., 2014). The intricate interaction of auxin and cytokinin in the provasculature of the embryo is propagated in the mature vasculature. Auxin signalling maxima occur in the xylem axis (Bishopp et al., 2011b), while cytokinin response is highest in the phloem (Mähönen et al., 2006a). Cytokinin signalling regulates expression of certain *PIN* members in the procambial cells of *Arabidopsis.* Thereby auxin flux is directed to the xylem axis, which creates an auxin maximum in the protoxylem. Auxin in turn induces the expression of the cytokinin signalling inhibitor *AHP6*, hence restricting the cytokinin signalling domain (Bishopp et al., 2011b). In contrast, the HD-ZIPIII transcription factor PHABULOSA (PHB) represses *AHP6* in a dose-dependent manner in the xylem axis (Carlsbecker et al., 2010).
Cytokinins also play a role in cambial activity during secondary growth, which is responsible for stem thickening. In poplar, reduction of endogenous cytokinin levels by misexpression of a *CKX* was shown to decrease radial expansion (Nieminen et al., 2008). Similar observations were made in an *Arabidopsis ipt1,3,5,7* mutant (Matsumoto-Kitano et al., 2008; Miyashima et al., 2013).

CYTOKININ IN APICAL MERISTEMS

The meristems at the two tips of the plant harbour the stem cells that maintain postembryonic growth. Cytokinin adopts outright opposite roles in the two meristems; in the RAM, cytokinin drives cell differentiation, while driving cell division in the SAM. Accordingly, auxin generally opposes cytokinin function in the RAM while rather supporting it in the SAM.

Root apical meristem

Postembryonic root growth is maintained via the activity of the RAM. The root apex is subdivided into the proximal meristem, a transition zone (TZ), and an elongation zone (EZ) (Petricka et al., 2012). Cells in the proximal meristem undergo mitotic divisions, increasing cell number in the root. As they are displaced by succeeding cells, they enter the TZ in which they stop dividing. Finally, cells start elongating and differentiating in the EZ. The RAM represents a paradigm to illustrate auxin-cytokinin antagonism: auxin promotes cell division in the proximal meristem while cytokinin induces cell differentiation in the transition zone (TZ) (Dello Ioio et al., 2007; Schaller et al., 2015). The antagonistic actions between auxin and cytokinin are mediated through the regulation of *SHY2*. SHY2 is an Aux/IAA protein, thus a repressor of auxin target genes. Cytokinin directly activates expression of *SHY2* via the type-*B* ARR1 in the vascular TZ. Induction of *SHY2* in turn causes downregulation of *PIN* expression, which limits auxin distribution (Dello Ioio et al., 2008; Růžička et al., 2009). Besides affecting auxin efflux, elevated cytokinin levels have also been found to dampen expression of *LAX1*, encoding an auxin influx carrier protein. This occurs mainly in the root vasculature (Zhang et al., 2013). Meanwhile, in areas with increased auxin, SHY2 is targeted for degradation via the SCF^{TIR1} ubiquitin-ligase complex (Gray et al., 2001). Intriguingly, SHY2 negatively

regulates biosynthesis of cytokinin by downregulation of *IPT5* (Dello Ioio et al., 2008). During the establishment of the meristem, the cell division rate must exceed cell differentiation rate to allow meristem growth. During this phase, the 5 days after germination, *ARR1* is repressed by gibberellin and hence also *SHY2* levels are low. Basal expression of *SHY2* is mediated through another *B*-type transcription factor, ARR12. Once gibberellin levels decrease, *ARR1* levels rise and allow for increased expression of *SHY2*, which boosts differentiation (Moubayidin et al., 2010). Through the opposing actions of cytokinin and auxin on *SHY2*, sharp boundaries of mutually exclusive signalling domains can be created. Cytokinin-mediated redistribution of PINs in the RAM also occurs post-transcriptionally, in a *SHY2*-independent manner via the type-*A* ARRs (Zhang et al., 2011). In the quiescent centre (QC), a group of cells with a local auxin maximum and little mitotic activity (Dolan et al., 1993; Sabatini et al., 1999), cytokinin limits expression of the transcription factors *SCARECROW (SCR)* and *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)* in an *ARR1*- and *ARR12*-dependent manner (Zhang et al., 2013). *SCR* expression in the QC is known to be both necessary and sufficient for QC identity (Sabatini et al., 2003), while homeobox protein WOX5 is required non-autonomously to prevent stem cell differentiation (Sarkar et al., 2007). SCR, on the other hand, was shown to repress *ARR1* in the QC. ARR1, however, activates transcription of *ASB1*, an auxin biosynthesis gene and the elevated auxin level acts positively on the expression of *SCR* (Moubayidin et al., 2013). Altogether, this intricate reciprocal regulation ensures a robust set up in which a high auxin to cytokinin ratio specifies the QC.

Shoot apical meristem

Analogous to RAM function, the SAM drives continuous growth of the shoot. The SAM is characterized by its dome-shape at the centre of which lies the central zone (CZ) that spans the three cell layers L1, L2 and L3. L1, L2 and L3 give rise to the distinct cell types of the plant stem; epidermis, ground tissue and vasculature, respectively. The CZ is where the stem cells reside. They divide asymmetrically recreating stem cells on one hand and daughter cells that will eventually undergo differentiation on the other hand. Successive divisions push the daughter cells towards the peripheral zone (PZ), radially surrounding the CZ, and to the rib zone (RZ) below the stem cells. There, they undergo further rounds of division. Stem cell competence is maintained and induced via a so-called organizing centre (OC) that lies underneath the stem cells (Schoof et al., 2000).

In contrast to its role in promoting differentiation in the root apex, cytokinin induces proliferation of stem cells in the SAM. As mentioned earlier, a distinct cytokinin signal can be detected in heart-stage embryos at the prospective SAM (Zürcher et al., 2013), which coincides with expression of the stem cell marker *CLAVATA3* (*CLV3*) (Brand et al., 2002). *CLV3* expression depends on WUSCHEL (WUS), a homeodomain transcription factor that is necessary for SAM maintenance (Laux et al., 1996; Brand et al., 2002). *WUS* is expressed already during early embryogenesis, but in heart-stage embryos, distinct expression at the future SAM is observed (Tucker et al., 2008).

Postembryonically, high cytokinin activity can be detected in the OC (Laux et al., 1996) from where it acts largely in a non-cell autonomous fashion via WUS. Cytokinin induces expression of *WUS* in the OC from where the protein translocates via plasmodesmata into the CZ (Yadav et al., 2011; Daum et al., 2014). WUS represses *ARR7* and *ARR15* expression and thereby strengthens cytokinin signalling creating a positive feedback loop (Leibfried et al., 2005; Gordon et al., 2009). WUS that has moved into the CZ, induces *CLV3* which non-autonomously restricts *WUS* expression to the OC via the leucine-rich receptor (LRR) CLV1 (Fletcher et al., 1999; Brand et al., 2000; Yadav et al., 2011). Another transcription factor in the CZ, HECATE1 (HEC1), was recently discovered to indirectly limit *WUS* expression (Schuster et al., 2014). HEC1 is a basic helix-loop-helix (bHLH) transcription factor promoting cell division by inducing cell cycle genes. Additionally, HEC1 induces type-*A ARRs*, which negatively affects cytokinin-mediated *WUS* induction in the CZ. In the OC, *HEC1* expression is in turn suppressed by WUS (Schuster et al., 2014).

SHOOTMERISTEMLESS (STM), a second homeodomain transcription factor besides WUS acts positively on cytokinin production by inducing *IPT7* expression (Yanai et al., 2005; Scofield et al., 2013). Strong *stm* mutant alleles display shoot meristem loss at the end of embryogenesis. Expression of *IPT* under the *STM* promoter or application of exogenous cytokinin in *stm* mutants can partially rescue the phenotype indicating that induction of cytokinin biosynthesis is a main task of STM (Yanai et al., 2005). However, additional functions in SAM organization can be attributed to STM (Scofield et al., 2013). Cytokinin ribotides produced by IPT action need to be activated by LOG proteins. Localized expression of the *LOG4* gene in the L1 layer creates a gradient of active cytokinin in the SAM (Kuroha et al., 2009; Chickarmane et al., 2012). Still, the OC is the zone of highest cytokinin activity, an observation that can partially be explained by the expression pattern of the *AHK4* receptor; it is not significantly expressed in the upper layers of the SAM, hence, only little cytokinin response occurs there (Chickarmane et al., 2012). Contrary to counteracting cytokinin as in the RAM, auxin acts synergistically with cytokinin function in the SAM; it represses type-*A* ARRs *ARR7* and *ARR15* via the AUXIN RESPONSE FACTOR5 (ARF5)/MONOPTEROS (MP) thereby enhancing cytokinin sensitivity of the OC (Zhao et al., 2010).

LATERAL ROOT DEVELOPMENT

In order to optimally mine the soil, plants develop lateral roots (LR), which increases the surface at which nutrient exchange may occur. The process of LR development starts with a series of anticlinal divisions of xylem pole pericycle cells. Subsequent peri- and anticlinal divisions build the primordium that will form the emerged LR (Malamy and Benfey, 1997). LR organogenesis is known to rely on auxin transport (Casimiro et al., 2001). As it forms, auxin flux is redirected towards the growing tip of the new organ. This flux is mediated primarily by the auxin efflux carriers, PINs (Benková et al., 2003) and to a lesser extent, LAX3 (Péret et al., 2013). Cytokinin signalling represses the

formation of LR by directly affecting PIN distribution patterns during early stages of organogenesis (Laplaze et al., 2007; Marhavý et al., 2011). Accordingly, cytokinin is repressed during LR initiation, at least in part through the action of AHP6 (Moreira et al., 2013), and several cytokinin signalling mutants display increased numbers of LR (Werner et al., 2003; To et al., 2004; Chang et al., 2013). Later, cytokinin signalling causes degradation of PIN1 from anticlinal membranes to ensure auxin flux towards the new growing tip (Marhavý et al., 2014). Differences in the phosphorylation statuses of PIN1 at anticlinal versus periclinal membranes underlie the differential sensitivity towards cytokinin. This cytokinin-mediated lytic degradation of PIN1 was demonstrated to be independent of transcription and may therefore represent a rapid modulation of PIN1-dependent auxin flow (Marhavý et al., 2011, 2014). Spacing of the LRs on a root is influenced by cytokinin signalling maxima in pericycle cells along the root; between two emerged primordia, cytokinin response is largest and inhibits LR organogenesis (Bielach et al., 2012). Spatially regulated cytokinin synthesis is involved in producing these local peaks of cytokinin (Chang et al., 2015).

CONCLUDING REMARKS AND FUTURE CHALLENGES

The first cytokinin was isolated as the active substance that allowed sustained growth of cultured tissue. Many additional effects on plants were then discovered, and the elucidation of the core signalling pathway followed. Recent research has characterized the signalling mechanistic at a more detailed level providing high-resolution models of how cytokinin signalling integrates into the specific developmental context, including how it interconnects with other signals. The increasing level of detail in our understanding comes with increased awareness of the complexity of cytokinin signalling intimately connected with plant life. Obviously, plants are masters in integrating various signals to support pattern formation, growth and responses to a changing environment. Cytokinin takes one of the leading roles in the regulation of these processes, and plant researchers working on diverse topics will almost inevitably meet elements of cytokinin signalling while investigating plant strategies of successful survival and reproduction.

While core mechanisms of cytokinin signalling have been elucidated, there are questions remaining unanswered. The occurrence of a great number of homologues for virtually any of the components involved in biosynthesis, degradation and signalling implies intricate regulation of specificities, activities and functions. How, where and when individual family members are being regulated is not fully understood. Also, levels of active cytokinins are tightly regulated by synthesis, degradation and modifications. How homeostasis is regulated, and the role of modified cytokinin variants have not been scrutinized to date.

Biosynthesis and signalling activities do not coincide spatially, which indicates a transporting system for cytokinin. Specifically, producing cells need to export cytokinins, and we predict the existence of dedicated cytokinin exporters that await discovery.

While cytokinin has been implied in many developmental processes, additional functions, for example in early embryogenesis, regeneration, and functions in poorly characterized

plant species will be uncovered. Thus, despite the vast amount of accumulated data and knowledge, exciting revelations are to be expected, and we are curious to see how cytokinin research will continue to uncover more secrets of plant life.

1.2 UPDATES IN THE FIELD OF CYTOKININS

Since the writing of the book chapter, new discoveries were made in the still rapidly expanding field of cytokinin research. In this section, I want to shortly recapitulate some of these latest findings that broaden our knowledge of cytokinin action and regulation in specific contexts.

EMBRYO DEVELOPMENT

In angiosperms, sexual reproduction occurs through double fertilization, where one pollen fertilizes the egg cell giving rise to the embryo, while a second pollen fuses with the central cell to form the endosperm (Raghavan, 2003). A recent publication reported that plants mutant for *ALTERED MERISTEM PROGRAM1* (*AMP1*) show growth of supernumerary eggs due to misspecified synergids (Kong et al., 2015). *AMP1* encodes a putative glutamate carboxypeptidase that was proposed to influence expression of cytokinin metabolic or signalling genes (Chin-Atkins et al., 1996; Helliwell et al., 2001). A previous study on *amp1* had determined that cytokinin levels in seedlings at the rosette stage are significantly elevated in the mutant compared to the wild type (Chaudhury et al., 1993). However, in ovules of *amp1*, *TCSn*-tracked cytokinin signalling was not affected (Kong et al., 2015), leading to the conclusion that *AMP1* function in maintaining correct synergid differentiation is independent of type-B ARR-mediated cytokinin signalling. In contrast, it was reported that functional *CRFs* are required for embryo development as the quadruple mutant *crf1,2,5,6* turned out to be embryonic lethal (Raines et al., 2016). It would be interesting to know whether CRF-mediated cytokinin signalling output is affected in the *amp1* mutant, which could establish a link between the increased cytokinin levels and the misspecified synergids. Unfortunately, this aspect was not discussed by the authors in their report.

REPRODUCTIVE TISSUE DEVELOPMENT

As previously reported, application of exogenous cytokinin causes defects in gynoecium development (Zúñiga-Mayo et al., 2014) (see **Chapter 1.1**). It was now found that the developing flowers of the mutant combinations in the bHLH transcription factors *HECATE1* (*HEC1*), *HEC2*, *HEC3* or *SPATULA* (*SPT*) are hypersensitive to exogenous cytokinin (Schuster et al., 2015). These mutants showed apically unfused fruits and tissue proliferation after treatment with low BA concentrations that did not affect the wild type, indicating negative regulation of cytokinins by HECs and SPT. HEC1 was previously shown to induce type-*A ARRs* in the CZ (Schuster et al., 2014) (see **Chapter 1.1**) and it was therefore suggested (albeit not demonstrated) that HEC and SPT act through type-*A ARRs* also during fruit development. In addition to cytokinin modulation, HEC1 was found to act on auxin signalling by positively regulating *PIN1* expression (Schuster et al., 2015). In contrast, expression of the *TEOSINTE BRANCHED1-CYCLOIDEA- PCF15* (*TCP15*) transcription factor was shown to decrease auxin levels. Intriguingly, the overexpression

of *TCP15* also resulted in apically unfused fruits, indicating that auxin is a critical factor in gynoecium patterning. *TCP15* expression could be induced by cytokinin, and TCP15 in turn caused upregulation of the type-*A ARRs ARR7* and *ARR15* (Lucero et al., 2015) (Fig. 4a).

A recent study analysing the triple mutant of class III homeodomain leucine zipper (HD-ZIPIII) genes *CORONA* (*CNA*), *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) could show ectopic *WUS* expression in gynoecia. Gynoecium development has been reported to recapitulate organ development as it occurs in the SAM (Girin et al., 2009). Due to the known interdependency of cytokinin and *WUS* in the OC (see Chapter 1.1), the researchers assessed whether cytokinin upregulation could be the underlying cause for ectopic *WUS* expression in the *cna phb phv* mutant. Signalling levels as measured by the TCS::GFP marker were however not affected (Yamada et al., 2016), indicating that *CNA*, *PHB* and *PHV* confine *WUS* expression in gynoecium development independently of cytokinin.

APICAL MERISTEMS

Besides *SCR*, *SHORTROOT* (*SHR*) is known as a key regulator of root stem cell niche maintenance. Mutations in *SHR* cause terminate root growth due to differentiation of QC cells (Benfey et al., 1993; Helariutta et al., 2000; Xu et al., 2006), a phenotype that can be partially mimicked by exogenous application of cytokinin (Dello Ioio et al., 2007; Cui et al., 2011; Zhang et al., 2013). SHR negatively modulates *PHB* levels via induction of the microRNA *miR165/6* (Carlsbecker et al., 2010). In the *shr* mutant, high levels of *PHB* were found to cause upregulation of *IPT3* and *IPT7*, which concomitantly increased levels of cytokinins (Cui et al., 2011; Sebastian et al., 2015). Counter-intuitively, this increase was accompanied by decreased signalling output (Sebastian et al., 2015). Additional loss of *PHB* function in the *phb shr* double mutant restored normal root growth and normalized *IPT3* and *IPT7* transcript levels. However, cytokinin concentrations remained high, and signalling induction in *phb shr* reached higher levels than wild-type. Consistently, it could be shown that under high cytokinin levels, *PHB* function is also required to negatively regulate type-*B* ARR-dependent target gene activation (Sebastian et al., 2015). The persistent high levels of cytokinins in *phb shr* can be partly explained through parallel SHR-dependent induction of *CKX3*, which has been previously reported (Cui et al., 2011) (Fig. 4b). Interestingly, the root growth rescue in *phb shr* occurs without restoring the QC cells' identity (Sebastian et al., 2015), indicating that cytokinin regulates root growth and meristem size independently of the QC.

Analogously, *SCR*-mediated control of root meristem size was recently reported to occur not only through QC-localized SCR but also through *SCR* expression from the endodermis (Moubayidin et al., 2016). As mentioned earlier, the establishment of the root meristem is characterized by high cell division and low differentiation rate, which requires downregulation of *ARR1* through gibberellin (Moubayidin et al., 2010) (see Chapter 1.1). Furthermore, it was shown that SCR inhibits *ARR1* expression in the QC and that QC-localized *SCR* expression is required to limit *ARR1* expression to the TZ (Moubayidin et

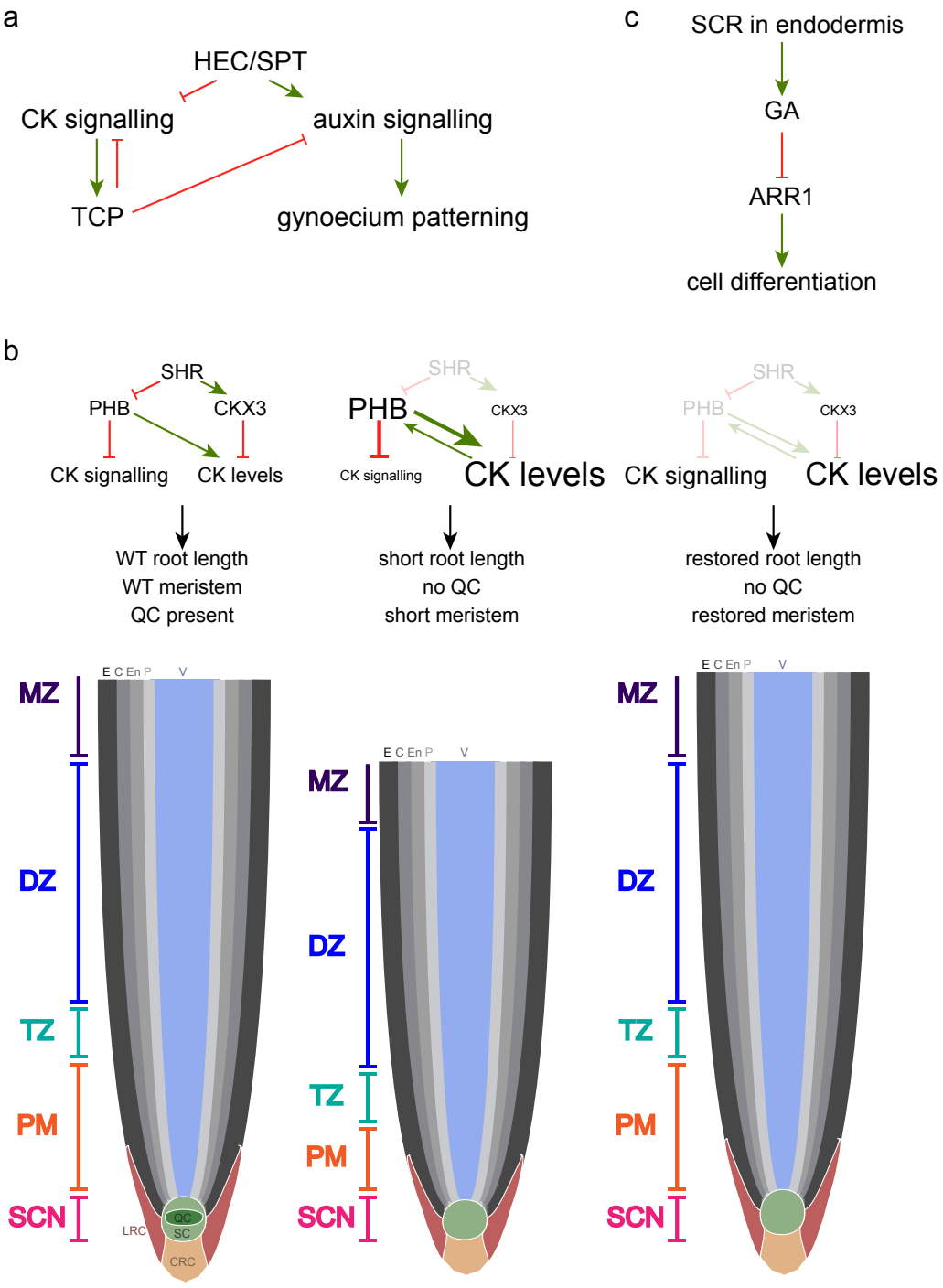

Fig. 4 | Simplified regulatory networks involved in gynoecium patterning and meristem size regulation in the RAM. a) TCP15 and presumably the HEC/SPT module act negatively on cytokinin signalling via induction of type-*A ARRs*. b) SHR reduces PHB levels in the vasculature via *miR165/6*. PHB enhances cytokinin levels through induction of *IPT3* and *IPT7* expression, but inhibits type-B ARR- dependent target gene activation. The *shr* mutant is characterized by a short root, short meristem and loss of QC (middle). The loss of *PHB* in the *shr* background restores root length and meristem size but does not restore QC (right). Addition of exogenous cytokinin can mimick the shr phenotype. c) Endodermally localized SCR enhances GA signalling through control of RGA. ARR1 promotes cell differentiation via SHY2.
SHR is expressed in the QC, endodermis and vasculature of the root tip; *SCR* is expressed in the QC and in the endodermis of the root tip; *PHB* is expressed in the stele, *ARR1* is expressed at the TZ. SCN = stem cell niche, PM = proximal meristem, TZ = transition zone, DZ = differentiation zone, E = epidermis, C = cortex, En = endodermis, P = pericycle, V = vasculature, CRC = columella root cap, LRC = lateral root cap, SC = stem cells, QC = quiescent centre, GA = gibberellic acid, CK = cytokinin. See text for gene acronyms.

al., 2013). It was now demonstrated that this regulation is reinforced by endodermal *SCR* acting upstream of gibberellin (Moubayidin et al., 2016). Endodermal SCR modulates levels of the DELLA protein REPRESSOR OF ga1-3 (RGA), which is a negative regulator of gibberellin signalling (Locascio et al., 2013). RGA downregulation through SCR allows gibberellin accumulation, which in turn restricts *ARR1* expression to the TZ (Moubayidin et al., 2016) (Fig. 4c).

Only lately, it was reported that the RAM of the quadruple and triple mutants *crf1,3,5,6* and *crf2,5,6* shows a reduced size and compromised root growth (Raines et al., 2016). In the shoot, rosette size of *CRF1*-, *CRF3*- or *CRF5*- overexpressing plants was reduced, whereas the triple mutants *crf1,5,6*, *crf2,5,6* and *crf3,5,6* had larger rosettes. Because cytokinin function is usually correlated with promoted shoot development and decreased root growth, these observations were taken as indicator for a negative role of redundantly acting *CRFs* in cytokinin signalling (Raines et al., 2016).

Concerning cytokinin action at the SAM, efforts were made to describe positioning of the OC by modelling. Integrating the current knowledge of cytokinin perception through AHK4, phosphorelay signalling, and cytokinin-*WUS*-*CLV3* feedback regulation, the model suggests that specification and positioning of the OC within the SAM is a result of patterning mediated by cytokinin signalling and self-organization (Adibi et al., 2016).

CYTOKININ INTRA- AND EXTRACELLULAR DISTRIBUTION

The different cytokinin metabolic enzymes are targeted to various subcellular locations (see Chapter 1.1). With such strong compartmentalization, it is not clear how the different cytokinin species themselves are distributed. To address this question, researchers have recently measured extracellular, intracellular and vacuolar cytokinins in *Arabidopsis* (Jiskrová et al., 2016). They could show that 90 % of the total cytokinin content in mature *Arabidopsis* leaves is located in the extracellular space, while 7 % are vacuolar and 3 % intracellular. Overall, inactive cytokinin types dominated over the active free bases, in line with the moderate requirement for differentiation or division processes in mature leaves. tZ and its derivatives as well as the N^7- and N^9-glucosides were found to occur primarily in the extracellular space, whereas iP located intracellularly but not to the vacuole (Jiskrová et al., 2016).

Measurements of cytokinins in seedling root symplast and apoplast showed that the free bases and ribosides are present in the apoplast fraction, whereas cytokinin glucosides were detected mostly in the intracellular space (Antoniadi et al., 2016, *personal communication*, March 2016). Together, these data suggest that the extra-, intra- and subcellular localization is dynamically regulated reflecting the context's requirements for cytokinin signalling activity.

1.3 CYTOKININ TRANSPORT

Already early studies acknowledged systemic cytokinin distribution through acropetal and basipetal transport of cytokinins via the xylem and phloem, respectively (Staden and Davey, 1979; Vonk and Davelaar, 1981; Bangerth, 1994; Beveridge et al., 1997). The pea *ramosus4* (*rms4*) mutant and the corresponding mutant in *Arabidopsis*, *more axillary branches* (*max2*), display decreased xylem cytokinin concentration (Beveridge et al., 1997; Foo et al., 2007). *MAX2* encodes a protein of the F-box leucine-rich repeat family (Stirnberg et al., 2002) and its mRNA was reported to travel basipetally through the phloem (Thieme et al., 2015), which could explain why mutant shoot stocks caused a mutant phenotype on wild-type root stocks in grafting experiments (Beveridge et al., 1997; Foo et al., 2007). The predicted function for *MAX2* lies in the perception of strigolactone, a phytohormone that modulates shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008; Hamiaux et al., 2012; Zwanenburg et al., 2016). Despite the discernible interaction between strigolactone and cytokinins, it has not been established how loss of *MAX2* function in the shoot eventually causes altered xylem cytokinin concentration. Nonetheless, the phenotype suggests that root-derived cytokinins have a role in orchestrating shoot architecture.
Further evidence supporting regulated root-to-shoot cytokinin transport comes from the *atabcg14* mutant. *AtABCG14* was characterized as transporter in the root pericycle that mediates xylem-loading of tZ. Loss of *AtABCG14* function led to reduced xylem cytokinin levels and stunted shoot growth (Ko et al., 2014; Zhang et al., 2014), supporting the notion that root-borne tZ is required in the shoot for normal growth.
Vice versa, transport of shoot-derived cytokinins through the phloem was shown to regulate root vascular patterning (Bishopp et al., 2011c). Reduction of cytokinin levels in the phloem led to altered procambial *PIN7* expression and aberrant xylem specification, indicating that the auxin-cytokinin interaction during vasculature development (Bishopp et al., 2011b) (see Chapter 1.1) is dependent on shoot-borne cytokinins.

The family of *ENTs* (see Chapter 1.1) and the family of *PUPs* are transporters that have been implicated in short-range cytokinin translocation (Gillissen et al., 2000; Bürkle et al., 2003; Hirose et al., 2005, 2008). *Arabidopsis* PUP1 was shown to mediate energy-dependent uptake of adenine and its derivatives into yeast and *Arabidopsis* cell cultures (Bürkle et al., 2003). In a similar study, vitamin B6 uptake could be furthermore attributed to PUP1, PUP2, PUP3 and PUP4 (Szydlowski et al., 2013). PUP1 was found to localize to the plasma membrane and to be expressed in hydathodes, from where it was suggested to function in the retrieval of substrates (Bürkle et al., 2003; Szydlowski et al., 2013). In tobacco, the *PUP*-like NICOTINE UPTAKE PERMEASE1 (NUP1) was shown to specifically import nicotine. NUP1 localizes to the plasma membrane and is expressed in roots. Its downregulation caused reductions not only in root but also shoot nicotine levels, and was accompanied by increased root elongation. However, competition with kinetin inhibited nicotine uptake only to a small extent indicating that NUP1's primary substrates are alkaloids rather than purine-derivatives (Hildreth et al., 2011). Later, it was shown that

NUP1 also allows transport of vitamin B6 and other pyridine-containing compounds (Kato et al., 2015), demonstrating that PUP1 and NUP1 exhibit partially overlapping substrate specificities. In rice, a mutant in *OsPUP7* was described to display increased size, fewer spikelets, longer leaves and delayed flowering (Qi and Xiong, 2013). Additionally, seed and grain length were increased compared to the wild type, a phenotype that is also observed in natural variants with decreased *OsCKX2* expression (Ashikari et al., 2005). In young spikelets of *ospup7*, levels of iP and iP riboside were elevated indicating that efflux of cytokinin from young spikelets, a major source of cytokinin, is impaired (Qi and Xiong, 2013).

Possibly owing to its high number of members, the *PUP* family was only poorly characterized to date. Apart from the study on *OsPUP7*, functional analyses using mutants have not been carried out and the role of *PUPs* in cytokinin homeostasis or plant development has not been assessed (Kudo et al., 2010). Their demonstrated capacity of transporting cytokinins makes them likely candidates for short-range transport that could underlie the observed specific spatiotemporal distribution of cytokinins. This thesis was therefore dedicated to the characterization of the *Arabidopsis* family of *PUPs* as candidates for a cytokinin transport system.

1.4 SCOPE OF THIS THESIS

Cytokinin represents a key hormone in the control of growth and development. Due to its potency to induce cell division and dell differentiation, the sites of cytokinin perception need to be under tight spatial and temporal regulation. Multiple evidence suggest that such spatiotemporal control may be achieved by the differential subcellular distribution of active cytokinin ligands mediated by the action of cytokinin transporters. Members of the *Arabidopsis* family of *PURINE PERMEASES* (PUP) were found to import cytokinin and other adenine derivatives in a heterologous yeast system and in *Arabidopsis* cell cultures (Gillissen et al., 2000; Bürkle et al., 2003). However, a role for the characterized members in shaping cytokinin signalling landscapes was not shown. In this thesis, I aimed at characterizing additional *PUP* genes in order to identify members that can modulate locales of cytokinin activity.

Cytokinin signalling activity initiates a multistep phosphorelay system, which culminates in the induction of target genes. The promoters of many of these target genes carry a consensus binding motif (Sakai et al., 2000; Hosoda et al., 2002), that was employed to design the synthetic reporter Two Component signalling Sensor (*TCS*) that allows *in vivo* spatiotemporal tracking of cytokinin signalling activities (Müller and Sheen, 2008). In a first part of the thesis, we have optimized the previously developed *TCS* reporter to obtain *TCSn*, a more robust and sensitive version with altered spacing, and sequence variation in the arrangement of binding sites (Zürcher et al., 2013). The use of the *TCSn* reporter allowed us to screen members of the *PUP* family whose deregulated expression altered signalling output.

Members of the plant-specific *PUP* family were characterized in regards to their expression levels, expression patterns and functions in cytokinin signalling. Differing expression patterns and transport capacities support sub- or neofunctionalization of *PUP* members. Most strikingly however, we could establish *PUP14* as a crucial determinant of cytokinin signalling landscape. It is expressed in a broad range of tissues and inducible RNAi knockdown lines of *PUP14* showed ectopic cytokinin signalling in many developmental contexts, which was furthermore accompanied by cytokinin-related morphological defects. PUP14 functions to transport bioactive cytokinins from the apoplast into the cell whereby it attenuates cytokinin perception. These findings postulate a previously unknown mechanism of cytokinin signalling regulation through differential translocation of active ligands, establishing the *PUP* family as modulators of signalling landscapes.

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2 Results TCSn

A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signalling network *in planta*

Cytokinin signalling occurs through a two-component phosphorelay that typically results in the activated expression of target genes. The nuclear type-B ARRs recognize a cognate *cis*-regulatory element in the promoter of target genes, which was exploited to design a synthetic reporter construct that allows the tracking of cytokinin signalling activities *in planta*. The following chapter describes how this synthetic reporter was optimized to yield a robust but sensitive signalling sensor.

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Author Contributions

BM devised experiments, cloned all constructs, generated transgenic plants, did microscopic imaging and wrote the manuscript. EZ did protoplast transactivation assays and wrote the manuscript. DT-D wrote the manuscript. KE did protoplast transactivation assays, PTT did microscopic imaging.

Please find the Supplementary Data online on
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A Robust and Sensitive Synthetic Sensor to Monitor the Transcriptional Output of the Cytokinin Signaling Network in Planta[1][C][W][OA]

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Cytokinins are classic plant hormones that orchestrate plant growth, development, and physiology. They affect gene expression in target cells by activating a multistep phosphorelay network. Type-B response regulators, acting as transcriptional activators, mediate the final step in the signaling cascade. Previously, we have introduced a synthetic reporter, *Two Component signaling Sensor* (*TCS*)::*green fluorescent protein* (*GFP*), which reflects the transcriptional activity of type-B response regulators. *TCS*::*GFP* was instrumental in uncovering roles of cytokinin and deepening our understanding of existing functions. However, *TCS*-mediated expression of reporters is weak in some developmental contexts where cytokinin signaling has a documented role, such as in the shoot apical meristem or in the vasculature of Arabidopsis (*Arabidopsis thaliana*). We also observed that GFP expression becomes rapidly silenced in *TCS*::*GFP* transgenic plants. Here, we present an improved version of the reporter, *TCS new* (*TCSn*), which, compared with *TCS*, is more sensitive to phosphorelay signaling in Arabidopsis and maize (*Zea mays*) cellular assays while retaining its specificity. Transgenic Arabidopsis *TCSn*::*GFP* plants exhibit strong and dynamic GFP expression patterns consistent with known cytokinin functions. In addition, GFP expression has been stable over generations, allowing for crosses with different genetic backgrounds. Thus, *TCSn* represents a significant improvement to report the transcriptional output profile of phosphorelay signaling networks in Arabidopsis, maize, and likely other plants that display common response regulator DNA-binding specificities.

The plant hormone cytokinin comprises a class of small, adenine-derived organic molecules that influence plant development and physiology in diverse contexts throughout the plant life cycle. Cytokinins initiate a multistep phosphorelay (MSP) signaling cascade by binding to and activating the cognate receptors, hybrid kinases with a cyclases/His kinases-associated sensory extracellular ligand-binding domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). In Arabidopsis (*Arabidopsis thaliana*), these are encoded by the *ARABIDOPSIS HIS KINASE2* (*AHK2*), *AHK3*, and *AHK4* genes. Ligand binding triggers autophosphorylation at a conserved His residue in the receiver domain and subsequent transfer of the phosphoryl group to a conserved Asp residue in the attached transmitter domain. Besides the cytokinin receptors, eight other hybrid kinases are encoded by the Arabidopsis genome, including *CYTOKININ INDEPENDENT1* (*CKI1*), which can potentially activate the MSP signaling network. From the Asp of the hybrid kinase, the phosphoryl group is passed on to one of five ARABIDOPSIS HIS PHOSPHOTRANSFER proteins and then to a nuclear ARABIDOPSIS RESPONSE REGULATOR (ARR), of which there are type-A, type-B, and type-C. Members of the type-B class bind to promoters of target genes via their Myb-like DNA-binding domain and activate transcription, while type-A and type-C ARRs inhibit signaling activity. At the same time, type-A ARRs are immediate-early target genes of activated type-B ARR proteins, which establishes a negative feedback loop to the signaling pathway (Werner and Schmülling, 2009; Argueso et al., 2010; Perilli et al., 2010; Bishopp et al., 2011a; Hwang et al., 2012).

Despite the apparent simplicity of the MSP signaling mechanism, the precise identification and functional characterization of the diverse signaling locales poses several challenges. First, the distribution of active cytokinin ligands in planta is difficult to determine. Cytokinins are produced by complex enzymatic biosynthetic pathways in different cellular compartments and are

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subject to long- and short-range transport and degradation (Werner et al., 2006; Hirose et al., 2008; Bishopp et al., 2011b). Although distribution patterns of cytokinins using monoclonal antibodies have been reported (Aloni et al., 2004, 2005), the available antibodies detect only a subset of active cytokinins, as well as inactive precursor forms (Eberle et al., 1986). Besides AHK2, AHK3, and AHK4, cytokinin-independent hybrid kinases, in particular CKI1 (Pischke et al., 2002; Hejátko et al., 2003, 2009; Deng et al., 2010), but potentially also the ethylene receptor ETR1 (Cho and Yoo, 2007; Hall et al., 2012) or AHK5 (Mira-Rodado et al., 2012; Pham et al., 2012), can activate the MSP network. The use of mutants is complicated because of redundantly acting signaling components, which require the generation of higher order mutants. For many gene families, these are difficult or impractical to generate due to the high number of genes involved, the lack of null mutants, or the close linkage of loci. Moreover, phenotypes caused by a loss of signaling are often pleiotropic or cause early lethality, which can mask functions of interest. In contrast to these difficulties, visualizing the transcriptional MSP output with a synthetic reporter reveals the sites of action during wild-type development. This information then allows focusing on the specific context for functional analyses, such as applying targeted genetic approaches or chemical and pharmacological treatments, and tracking the immediate consequences on the signaling output. The Myb-like DNA-binding domain of the 11 different type-B ARR family members is conserved, in particular, in the nine residues that were shown to make direct DNA contact (Hosoda et al., 2002). Accordingly, in vitro binding studies with the DNA-binding domains of different type-B ARRs identified very similar binding specificities, with the consensus sequence 5′-(A/G)GAT(C/T)-3′ (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). This apparent similarity in the DNA binding specificity of the different type-B ARR family members was exploited to design a specific synthetic sensor, *Two Component signaling Sensor* (*TCS*), which is based on concatemeric 5′-(A/G)GAT(C/T)-3′ binding sites (Fig. 1, A–D; Müller and Sheen, 2008). In transgenic *TCS*::*GFP* plants, the *GFP* signal reflecting the signaling output pattern has facilitated describing novel cytokinin functions (Müller and Sheen, 2008; Bencivenga et al., 2012; Marsch-Martínez et al., 2012), as well as refining and deepening the understanding of existing cytokinin functions (Leibfried et al., 2005; Gordon et al., 2009; Zhao et al., 2010; Bielach et al., 2012; Chickarmane et al., 2012; Murray et al., 2012). Despite the documented value of *TCS*-controlled reporters, some limitations emerged, which motivated us to construct an improved version. First, *TCS*-induced expression is weak in certain developmental contexts where cytokinin signaling has a documented role, such as in the embryo sac (Pischke et al., 2002; Hejátko et al., 2003; Deng et al., 2010; Bencivenga et al., 2012), in the shoot (Gordon et al., 2009; Zhao et al., 2010; Chickarmane et al., 2012), and in the vasculature (Mähönen et al., 2000, 2006a, 2006b; Dello Ioio et al., 2008). Second, we observed that GFP expression becomes progressively reduced with increasing generations, such as in the root meristem of the seedling (Fig. 1B), presumably due to silencing effects triggered by the monotony of the repetitive sequence in *TCS* (Chan et al., 2005). Here, we present a superior version, *TCS new* (*TCSn*)::*GFP*, which exhibits higher sensitivity to cytokinin and MSP components in transient transfection assays (Fig. 2) and a much brighter GFP signal in most tissues analyzed (Figs. 3 and 4). Thus, the *TCSn*::*GFP* expression pattern reveals aspects of the MSP output that were not reported by *TCS*::*GFP*. Furthermore, GFP expression has been stable during propagation, indicating that unlike *TCS*::*GFP*, it does not easily get silenced.

RESULTS

Defining Relevant Parameters to Improve TCS Activity

To reliably and consistently monitor low-to-intermediate output levels of the MSP network in planta and to avoid transgene silencing, we sought to improve the current synthetic sensor TCS (Müller and Sheen, 2008). Its design is based on the in vitro-defined DNA consensus sequence 5′-(A/G)GAT(C/T)-3′, as recognized by type-B ARRs (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). To identify parameters that affect the activity of TCS, derivatives were constructed with variations in the number of binding sites, phasing, and identity of flanking nucleotides. All of the resulting fragments were cloned upstream of the cauliflower mosaic virus minimal 35S promoter and transcriptionally fused to luciferase (LUC). The ability of these constructs to confer cytokinin-dependent transcriptional activation was experimentally tested in transient transfection assays of primary mesophyll protoplasts (Müller and Sheen, 2008). An oligonucleotide harboring four such bindings sites, separated by arbitrarily selected flanking nucleotides, represented the basic building block for *TCS* (Fig. 1C). Multimerization of this sequence fragment resulted in various derivatives with an increasing number of binding sites (Fig. 1A). The arrangement of binding sites was chosen to realize all possible orientations that two given motifs can have relative to each other: tandem, tail to tail, and head to head (Fig. 1, A and D). Minimal but robust activity was observed with eight binding sites, while 16 sites resulted in a sensitivity comparable to *ARR6*::*LUC*, a reporter based on the 5′ cis-regulatory region of *ARR6* (Hwang and Sheen, 2001), a type-A *ARR* with 11 clustered 5′-(A/G)GAT(C/T)-3′ motifs in its promoter (Fig. 1A; Supplemental Table S2). A dramatic increase in cytokinin responsiveness occurred when the number of sites increased from 16 to 24. Such a sigmoidal response curve is indicative of synergistic interactions among activator binding sites (Carey, 1998). The addition of more sites did not stimulate the activity further (Fig. 1A). Thus, 24 binding sites were chosen for the final design, which was named TCS (Müller and Sheen, 2008). A variant of *TCS*,

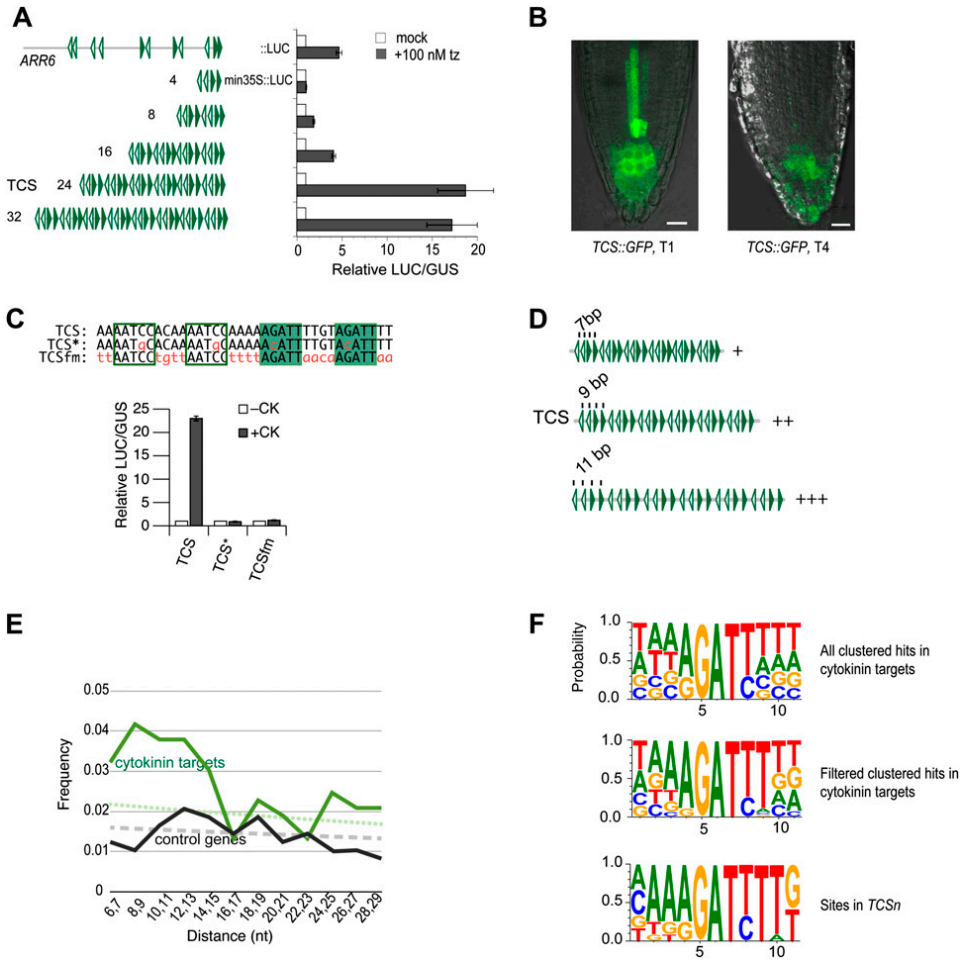

Figure 1. Optimization of *TCS*. A, A concatemer of 24 repeats of the 5′-(A/G)GAT(C/T)T-3′ binding motif caused the strongest cytokinin-dependent induction of a LUC reporter in transient transfection assays. B, Reduced GFP signal in the primary root meristem of a 5-d-old transgenic *TCS::GFP* seedling in the fourth generation (T4) compared with a primary transformant (T1). C, Similar to mutating nucleotides essential for in vitro binding of type-B ARRs (*TCS*::LUC*, where the asterisk indicates point mutation G→C), the mutation of flanking nucleotides (*TCSfm*::*LUC*) abolished cytokinin-dependent response of *TCS*::*LUC*. D, Scheme representing the quantitative effects of different phasings of core 5′-(A/G)GAT(C/T)T-3′ motifs. Phasing of 11 bp results in strongest reporter gene expression. E, The frequency of 5′-(A/G)GAT(C/T)T-3′ motifs with a distance of 7 to 15 bp in cytokinin target genes (top curve) is higher than expected (dotted line), while the same motifs are not significantly higher than expected in control genes (lower curve and dashed line). F, Sequence logos (Crooks et al., 2004) generated from the alignment of clustered 5′-(A/G)GAT(C/T)T-3′ motifs in cytokinin target genes as listed in Supplemental Table S2 (top) after filtering with 5′-A(A/G)GAT (C/T)T-3′ and 5′-A (A/G)GAT(C/T)TT-3′ (middle), and the alignment based on the 12 motifs used to construct *TCSn* as listed in Supplemental Table S4 (bottom). Filled or empty arrowheads (A and D) or boxes (C) indicate 5′-A (A/G)GAT(C/T)TT-3′ motifs on the forward or reverse DNA strand, respectively. Bars = 20 μm. [See online article for color version of this figure.]

TCSfm (for *flanking nucleotides mutated*), harbors mutations in the nucleotides that flank the in vitro-defined core sequences and was expected to integrate cytokinin-dependent induction similar to *TCS*, recapitulating the results of in vitro binding studies. Notably, *TCSfm* is insensitive to cytokinin (Fig. 1C), indicating that the in vitro-defined core motif 5′-(A/G)GAT(C/T)-3′ is too short to support transcriptional activation in vivo. Other derivatives of *TCS* differ in the distance between binding sites (Fig. 1D). Compared to *TCS* with a 9-bp distance between the core motifs, 11 bp resulted in higher activity, while reducing the distance to 7 bp caused a substantial reduction in activity (Fig. 1D). Eleven base pairs correspond approximately to one helical turn of the DNA double helix in its common B configuration (Wang, 1979). In agreement with our findings, helical phasing has been shown to be an important parameter for the functionality of transcription factor binding motifs in individual genes (Bouallaga et al., 2000; Mack et al., 2000; D'Alonzo et al., 2002) and has also been observed at a global scale (Ioshikhes et al., 1999; Makeev et al., 2003). Based on these experiments, we reasoned that improving *TCS* could be achieved by using extended type-B ARR binding motifs and adjusting the phasing of motifs to 11 bp.

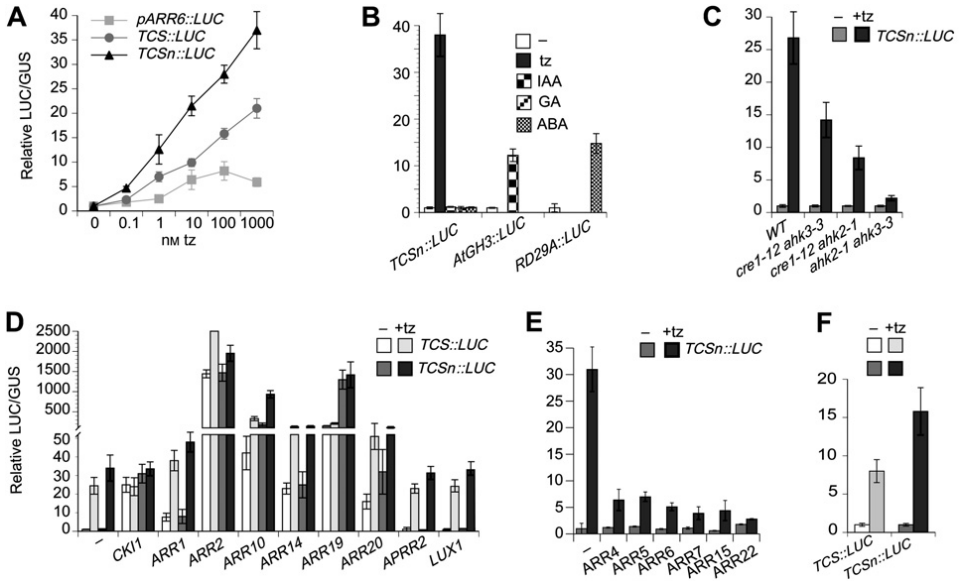

Figure 2. Sensitivity and specificity of *TCSn*::*LUC* in transient transfection assays. A, Induction of *ARR6*::*LUC*, *TCS*::*LUC*, and *TCSn*::*LUC* to increasing concentrations of transzeatin. B, *TCSn*::*LUC* is induced by transzeatin, but not by auxin, GA_3, or abscisic acid. *AtGH3*::*LUC* and *RD29A*::*LUC* serve as positive controls for auxin and abscisic acid hormone induction, respectively (Müller and Sheen, 2008). C, Cytokinin-dependent induction of *TCSn*::*LUC* is compromised in *ahk4 ahk3*, *ahk4 ahk2*, and *ahk2 ahk3* double mutant cells. *cre1-12* is a mutant allele of *AHK4* (Higuchi et al., 2004). D, Positive regulators of the MSP network induce *TCSn*::*LUC* expression. APRR2 and LUX have no effect. E, Type-A and type-C ARR attenuate cytokinin-dependent induction of *TCSn*::*LUC*. F, *TCS*::*LUC* and *TCSn*::*LUC* are induced in maize protoplasts by transzeatin. tz, Transzeatin; IAA, auxin; GA, GA_3; ABA, abscisic acid.

Bioinformatic Analyses to Identify Relevant Type-B ARR Consensus Binding Sites in Vivo

To analyze the type-B ARR binding motifs as they occur in vivo, we analyzed the sequence of the 10 type-A *ARR* genes (*ARR3*–*ARR9* and *AAR15*–*AAR17*). These genes represent the best-documented direct cytokinin target genes (D'Agostino et al., 2000; Taniguchi et al., 2007; Brenner et al., 2012). As a negative control, genes were randomly picked from a list of genes that showed stable expression irrespective of developmental stage, stress, and pharmacological or physiological treatments (Czechowski et al., 2005). Since the cis-regulatory sequence is typically found upstream, but can also be located the within transcribed sequence (Yant et al., 2010; Ritter et al., 2012), about 3 kb of the 5′-upstream sequence, as well as the transcribed sequence from each gene, was included in the analysis, totaling 45 kb of sequence for the 10 type-A *ARR* genes and 58 kb for the control genes (Supplemental Table S6). In each set, the number of hits to the 5′-(A/G)GAT(C/T)-3′ motif was counted. This motif has been shown to be indispensable for type-B *ARR* binding in vitro (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003) and for function in vivo (Ross et al., 2004; Müller and Sheen, 2008; Zhao et al., 2010; Liang et al., 2012). However, it is short and degenerate and thus occurs frequently by chance: on average, once in 108 bp of random DNA sequence. In the control genes, the frequency of motifs falls within a 95% confidence interval of a Poisson distribution, consistent with random frequency. By contrast, a hit is found every 86 bp in the cytokinin target genes. Such, or even higher, densities are very unlikely to occur by chance assuming Poisson distribution ($P < 10^{-7}$), suggesting that a considerable fraction of the 5′-(A/G)GAT(C/T)-3′ motifs found in cytokinin target genes is functional in integrating cytokinin input (Supplemental Table S1). Next, distances and relative orientations of these motifs were analyzed. Specifically, we measured the distances between two given hits and sorted them into different size classes. The motif itself measures 5 bp; thus, the shortest distance between two hits is 6 bp. Because we were mainly interested in clustered hits, we did not resolve distances greater than an arbitrary 30 bp. The expected size distribution follows the function: $F(n) = (1 - P)^{n-1}P$, where P is the probability to find a hit (Basler, 2000). In the cytokinin target genes, distances from 6 to 30 bp are significantly overrepresented (X^2 [1, n = 527] = 21.1, $P < 0.00001$), suggesting that these motifs are functional to support cooperativity of transcription factor binding in natural promoters, similar to what was observed in synthetic sequences (Fig. 1A). The observed enrichment concentrates to distances of 7 to 14 bp (Fig. 1E; Supplemental Table S3). When the relative orientation of clustered motifs was analyzed, no apparent bias either toward tandem or inverse orientations was detected (Supplemental Table S3). The analysis suggests that clustered 5′-(A/G)GAT(C/T)-3′ hits are

significantly enriched in cytokinin target genes, and we used these hits to create an alignment. An additional three nucleotides flanking the core site were included, totaling 11 nucleotides, which was determined as the optimal phasing based on transient transfection experiments reported above (Fig. 1D). The resulting alignment, represented by a sequence logo (Crooks et al., 2004), revealed the tendency for conservation of nucleotides flanking the core (Fig. 1F). Specifically, sequences accompanied by a 5′ extension, 5′-A-3′ and/or a 3′ extension, 5′-T-3′ appear more frequently, similarly to previous findings (Rashotte et al., 2003; Taniguchi et al., 2007). We used this information to reduce the number of nonspecific motifs from the alignment and filtered the list of motifs using the sequences 5′-A(A/G)GAT(C/T)-3′ and 5′-(A/G)GAT(C/T)T-3′ (Supplemental Table S2). The resulting smaller set of binding motifs yielded a sequence logo with a AA(A/G)GAT(C/T)TT consensus (Fig. 1F). This consensus was also found enriched in the cytokinin target genes compared with the control genes (not shown), similar to previous studies (Brenner et al., 2012).

Based on the refined sequence logo, we created 12 sites, each slightly different from the other. Their alignment creates a sequence logo, which is similar to the natural sites (Fig. 1F; Supplemental Table S4). These synthetic sites were combined in random order to result in a synthetic sequence fragment that was repeated once to harbor 24 binding sites (Supplemental Table S5). Since clustered sites in cytokinin targets show no preference for a specific relative orientation, we preserved the relative site arrangement of *TCS*, which supports all possible orientations (Fig. 1A). This improved synthetic fragment was named *TCSn*. To summarize, the results obtained from transient transfection assays combined with bioinformatic analyses of bona fide cytokinin target genes allowed us to construct a new synthetic cytokinin promoter with an optimized number, spacing, and sequence of motifs, while also including variations to reflect the range of potential diversity among sites found in natural targets. At the same time, sequence variations avoid sequence monotony that could trigger the silencing of *TCSn*::*GFP* in transgenic plants.

TCSn Specifically Integrates MSP Activity

Using transient transfection experiments of mesophyll protoplasts, *TCSn*::*LUC* was subjected to various assays to determine its sensitivity and specificity to MSP signaling (Fig. 2). Its sensitivity to cytokinin was higher compared with *TCS*::*LUC* or *ARR6*::*LUC* (Fig. 2A). Similar to *TCS* (Müller and Sheen, 2008), *TCSn* did not cause transcription of *LUC* upon incubation with the auxin indole-3-acetic acid, GA_3, or abscisic acid (Fig. 2B). Compared with wild-type cells, cytokinin-dependent expression of *TCSn*::*LUC* is compromised in cells that are mutated in two out of the three cytokinin receptor genes *AHK2*, *AHK3*, and *AHK4* (Fig. 2C). Cotransfection with positively acting signaling components, including CKI1 and type-B ARRs, stimulated *TCSn*::*LUC* expression. Notably, overexpression of ARR10, ARR19, and ARR20 caused markedly stronger induction of *TCSn*::*LUC* than of *TCS*::*LUC*, while the remaining type-B ARRs showed similar induction of both reporters. By contrast, ARABIDOPSIS PSEUDO-RESPONSE REGULATOR2 (APRR2) and LUX ARRHYTHMO (LUX), both of which share a very similar DNA-binding domain with type-B ARRs (Hwang et al., 2002; Helfer et al., 2011), do not activate *TCS* nor *TCSn* transcription (Fig. 2D). As expected, cotransfection with type-A ARRs attenuated cytokinin-dependent induction (Fig. 2E). Next, we tested the reporters in maize (*Zea mays*) mesophyll protoplasts. As could be predicted from the similarity of the type-B response regulators between monocots and dicots (Chu et al., 2011), *TCS*::*LUC* and *TCSn*::*LUC*

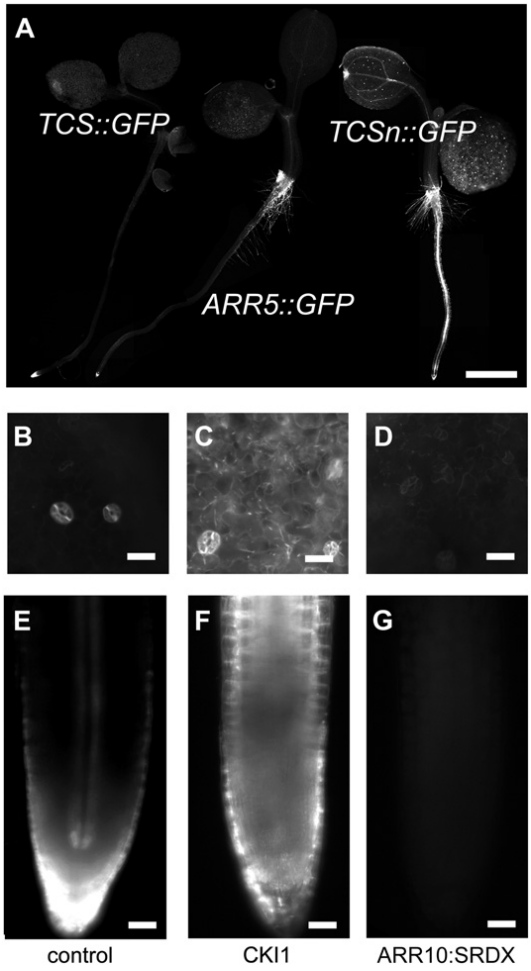

Figure 3. *TCSn*::*GFP* in the seedling. A, Compared with *TCS*::*GFP* and *ARR5*::*GFP*, *TCSn*::*GFP* exhibits strong GFP expression both in the root and shoot of the seedling. B to G, Induced overexpression of CKI1 (C and F) and ARR10:SRDX (D and G) causes ectopic activation or repression of *TCSn*::*GFP*, respectively, compared with controls (B and E) in the cotyledons (B–D) and the root meristem (E–G). Bars = 20 μm.

expression is activated by cytokinin in maize protoplasts (Fig. 2F). In summary, *TCSn* performs superior to *TCS* using protoplast transient assays, while retaining its specificity.

TCSn-Directed GFP Signals in the Seedling Are Brighter Than ARR5- or TCS-Controlled Reporters and Depend on MSP Signaling

Next, we analyzed the GFP expression pattern in plants that were transformed with a *TCSn*::*GFP* construct. Overall, *TCSn*::*GFP* expression levels are higher than *TCS*::*GFP* or *ARR5*::*GFP*, another frequently used cytokinin reporter (Fig. 3A). To address whether *TCSn*::*GFP* expression in planta is also controlled by MSP signaling, we transiently overexpressed proteins that either dominantly activate or repress MSP signaling. Compared with steady mutants, this approach avoids lethality issues and secondary effects. CKI1 induces MSP activity constitutively independent of cytokinins (Hwang and Sheen, 2001; Hejátko et al., 2009). Thus, *CKI1* expression was ubiquitously induced for 30 h in 3-d-old seedlings. Consequently, both in the main root tip and in epidermal cells of the cotyledons, ectopic and ubiquitous signaling activity is observed (Fig. 3, C and F), compared with the control (Fig. 3, B and E). By contrast, *TCSn*-dependent expression is affected by a dominant-negative version of the type-B ARR10. Specifically, ubiquitously expressed ARR10:SRDX caused a loss of the endogenous expression domains (Fig. 3, D and G). This result is in agreement with previous experiments where type-B ARRs harboring a chimeric repressor domain suppress phosphorelay signaling (Heyl et al., 2008; Müller and Sheen, 2008). Thus, ectopic activation of MSP signaling in planta induces *TCSn*::*GFP* expression, while dominant-negative interference with MSP output causes loss of the *TCSn*::*GFP* domains, indicating that *TCSn* specifically integrates MSP output.

TCSn::GFP Expression Patterns Correlate with Known Cytokinin Functions and Also Reveal New Functions

A detailed analysis of the *TCSn*::*GFP* expression patterns in different tissues revealed that the GFP activity is consistent with documented cytokinin functions. For example, during ovule primordia formation, *TCSn*-directed GFP expression, similar to *TCS*::*GFP* (Bencivenga et al., 2012), localizes to the basal part of the funiculus (Fig. 4I). However, while *TCS*::*GFP* expression levels are below the detection level at later stages, *TCSn*::*GFP* expression is visible till female gametophyte stage 7 of ovule development (Fig. 4K). A very weak signal is also detected in the nuclei of the female gametophyte nuclei at the micropylar pole of the embryo sac (Figure 4, J and K, arrows). During embryogenesis, *TCSn*::*GFP* expression is detected in the suspensor and later in the hypophysis. GFP expression is down-regulated in progenitors of the hypophysis defining the basal cell lineage, while a weak signal is detected in the lens-shaped cell, similar to *TCS*::*GFP* (Müller and Sheen, 2008). Notably, expression levels in suspensor and suspensor-derived cells is much weaker compared with *TCS*::*GFP*. By contrast,

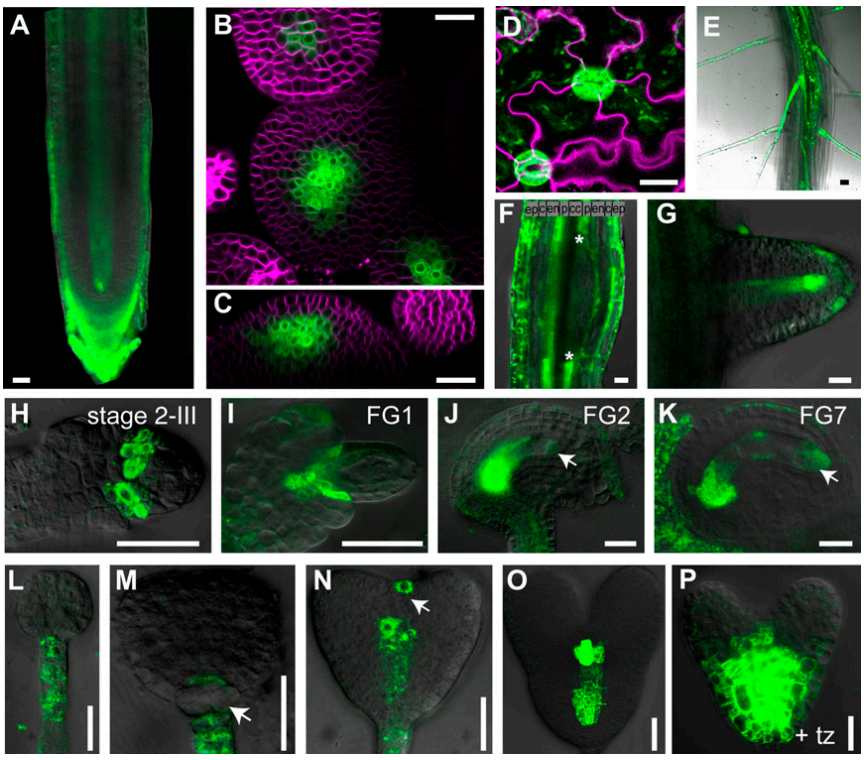

Figure 4. *TCSn*::*GFP* expression in different developmental contexts. A, Primary root meristem of 5-d-old seedling. B, Top view of shoot apical meristem. C, Side view of shoot apical meristem. D, Pavement cells and guard cells. E, Primary seedling root with root hairs. F, Lateral root primordium, early stage. Asterisks delineate lateral root primordium founder cells of pericycle that down-regulate MSP output. G, Emerging lateral root primordium. H, Ovule primordium after first mitotic division of megaspore mother cell stages, according to Schneitz et al. (1995). I to K, Embryo sac, stages according to Christensen et al. (1997). Arrows denote faint GFP signal in nuclei of embryo sac. L to P, Embryos. L, Globular stage. M, Transition stage, arrow denotes down-regulation of GFP in basal cell lineage. N, Heart stage, arrow denotes transient signal in the prospective shoot meristem. O, Late heart stage. P, Late heart stage, overnight incubation with 10 μM transzeatin. The signal from the membrane stain FM4-64 is shown in magenta. tz, Transzeatin; ep, epidermis; c, cortex; en, endodermis; p, pericycle cells; cc, central cylinder. Bars = 20 μm.

provascular cells and the prospective cells of the shoot meristem exhibit distinct and bright GFP signals (Fig. 4, N and O), which potentially allow for addressing novel cytokinin functions in these contexts. As expected, application of exogenous cytokinin leads to increased and expanded GFP expression (Fig. 4P). Expression in the columella cells of the root meristem in the seedling (Fig. 4A), in the vasculature of root (Figs. 3, A and E, and 4A), in the lateral root primordia (Fig. 4, F and G), in root hairs (Fig. 4E), in the shoot meristem (Fig. 4, B and C), in the shoot vasculature (Fig. 3A), and in pavement cells and guard cells (Fig. 4D) are qualitatively very similar to *TCS*::*GFP* (Müller and Sheen, 2008; Bielach et al., 2012; Chickarmane et al., 2012), but much stronger. Thus, in addition to revealing the peaks of cytokinin output, *TCSn*::*GFP* shows intermediate to low levels of signaling output as well. Furthermore, no reduction in GFP levels has been observed after three generations of selfing of transgenic plants (data not shown), indicating that unlike *TCS*::*GFP*, *TCSn*::*GFP* is not subject to transgene silencing.

DISCUSSION

Cytokinins activate a MSP network in target cells, which culminates in transfer of phosphoryl groups to type-B response regulators, nuclear proteins that specifically bind to DNA and activate transcription of selected target genes. Concatemeric binding motifs combined with a minimal promoter and transcriptionally fused with LUC or GFP resulted in a reporter that specifically mediates MSP output in vivo (Müller and Sheen, 2008). Ideally, activity mediated by a synthetic promoter reflects the pure and universal transcriptional output profile of the signaling activity, devoid of tissue-specific aspects or unrelated signaling input. Indeed, *TCS*-dependent expression patterns of GFP or GUS were useful in monitoring the specific sites of phosphorelay signaling output in different tissues, which guided the discovery of previously unknown cytokinin functions (Müller and Sheen, 2008; Bencivenga et al., 2012; Marsch-Martínez et al., 2012) and refined existing models of cytokinin function (Gordon et al., 2009; Zhao et al., 2010; Bielach et al., 2012; Chickarmane et al., 2012; Murray et al., 2012). However, *TCS*-mediated expression in planta is low in many contexts where MSP is known to be important, which motivated us to revise the design of *TCS* by optimizing and extending the binding motifs for the type-B ARRs. Furthermore, to counteract the silencing, we introduced sequence variations in nonessential nucleotides, which broke the monotony of the repetitive *TCS* sequence. These modifications resulted in *TCSn*, which, compared with *TCS*, demonstrates higher sensitivity to cytokinin (Fig. 2A) and a more balanced response to different type-B family members (Fig. 2D).

In transgenic plants, these improvements translate into increased GFP activity in all tissues analyzed (Figs. 3A and 4, A–K, N, and O), except for the suspensor and suspensor-derived cells of the embryo (Fig. 4, L–N). Qualitatively, the expression patterns of *TCS*::*GFP* and *TCSn*::*GFP* in planta are very similar during, for example, ovule primordia formation, embryogenesis, lateral root development, shoot meristem function, and vasculature formation (Figs. 3A and 4). The increased sensitivity of *TCSn*::*GFP* renders additional aspects of phosophorelay readout visible, which remained below the level of detection with *TCS*::*GFP*. Specifically, the expression domain in the shoot meristem is broader (Fig. 4, B and C; Gordon et al., 2009; Chickarmane et al., 2012), a GFP signal in pavement cells becomes visible (Fig. 4D), and a transient signal in the shoot meristem of the embryo at the heart stage is observed (Fig. 4N; Müller and Sheen, 2008). The apparent resistance against silencing will allow the crossing of the *TCSn*::*GFP* line in various genetic backgrounds with a reduced risk of decreased or variable GFP expression in the progeny. This will facilitate the analysis of phosphorelay signaling in many contexts, including the little-known roles in root hair development (Fig. 4E) or the emerging role in pavement (Fig. 4D; Li et al., 2012) and guard cells (Fig. 4D; Desikan et al., 2008; Mira-Rodado et al., 2012), among others.

Ideally, a synthetic reporter integrates the activities of all transcription factors involved in relaying the signal without bias toward specific family members. The 11 members of the type-B ARR family differ in their inherent activity levels, both in transient assays and in planta (Hwang and Sheen, 2001; Sakai et al., 2001; Imamura et al., 2003; Hass et al., 2004; Tajima et al., 2004; Heyl et al., 2008; Müller and Sheen, 2008; Kim et al., 2012; Liang et al., 2012). However, no reference exists for a given family member or relevant combinations thereof, and consequently, it is not clear how the ideal synthetic reporter would respond. Thus, while all type-B ARRs tested activate *TCS* and *TCSn* (Fig. 2D), we cannot exclude that the reporters may exhibit some bias toward specific type-B ARRs. For example, the low expression levels in the suspensor mediated by *TCSn*, compared with *TCS*, might reflect the better binding conditions for the set of type-B ARRs expressed in the suspensor, which facilitates integration of phosphorelay response; while in all other tissues, *TCSn* appears to provide better conditions.

Our experiments using an increasing number of binding elements in synthetic promoters suggest cooperative binding of the type-B ARRs (Fig. 1A). The statistical analysis of cytokinin target genes revealed an enrichment of clustered binding motifs, indicating that cooperative binding occurs in vivo as well. Further support for cooperative binding of type-B ARRs comes from the recent finding that ARR18 can homomerize in planta (Veerabagu et al., 2012). In prokaryotes, DNA-binding response regulators have been shown to dimerize and oligomerize (for review, see Galperin, 2006). Thus, in plants, an analogous model could be realized.

In summary, the improved sensor *TCSn* will allow a detailed study of MSP signaling in various

developmental contexts, shedding new light on plant development and physiology.

MATERIALS AND METHODS

Plant Growth and Treatments

Seedlings were germinated on vertical agar plates containing 1% (w/v) Suc, 0.8% (w/v) phytagar, and one-half-strength Murashige and Skoog medium with a 16-h/8-h photoperiod at 120 μmol m^{-2} s^{-1} and 21°C. After 3 d of growth on vertical plates, seedlings were transferred to liquid medium plates containing 1% (w/v) Suc, one-half-strength Murashige and Skoog medium, 2mM MES, pH 5.7, and 2% (v/v) ethanol for transgene induction in 12-well plates, sealed with parafilm, and incubated for 30 h before recording GFP fluorescence. Plants that needed to be grown to adulthood were kept in the greenhouse with a 16-h/8-h photoperiod at 150 μmol m^{-2} s^{-1} and 21°C during the light period and 18°C during the dark period. Plants used for protoplast transient transfection assays were grown with a 12-h/12-h photoperiod at 90 μmol m^{-2} s^{-1} and 21°C during the light period and 18°C during the dark period. Embryo in vitro cultivation and hormone treatments with transzeatin were performed as described (Müller and Sheen, 2008).

Plant Materials and Reporter and Effector Constructs

The *TCS*::*GFP* reporter line and the expression plasmids used in transient transfection assays were previously described (Hwang and Sheen, 2001; Yoo et al., 2007; Müller and Sheen, 2008). *LUX*, *APRR2*, and *ARR19* coding sequences were cloned into the expression vector as described (Hwang and Sheen, 2001). The *ARR5*::*GFP* reporter is composed of 2.3 kb of upstream regulatory sequence transcriptionally fused to GFP that localizes to the endoplasmatic reticulum (Ottenschläger et al., 2003) in the binary vector pCB302 conferring Basta resistance (Xiang et al., 1999). Plants carrying the inducible transgene *RIBOSOMAL PROTEIN S5A*::*AlcR*/*AlcA*::*ARR10*:SRDX are described in Müller and Sheen (2008). For the *35S*::*AlcR*/*AlcA*::*CKI1* transgene, the DM7 vector conferring kanamycin resistance (Müller and Sheen, 2008) was modified with a ligation-independent cloning adaptor annealed into linearized DM7 vector, digested with *Eco*RI and *Sal*I, to allow ligation of a *CKI1* PCR fragment, amplified from genomic ecotype Columbia DNA, which covers the *CKI1* locus from start to stop codons (see Supplemental Table S5 for sequences of the oligonucleotides). The *TCSn* plasmids *TCSn*::*GFP* and *TCSn*::*LUC* differ from the *TCS* variants (Müller and Sheen, 2008) by the synthetic promoter sequence. Plasmid and oligonucleotide sequences are provided in Supplemental Table S5. All plasmids have been sequenced to ensure no unwanted mutations have been introduced during cloning.

Phenotypic Analysis and Microscopy

GFP expression patterns (Fig. 3) were recorded using a Leica DM6000 microscope equipped with epifluorescence and a Leica DFC350FX camera, or with a Leica SP2 laser scanning confocal microscope (Fig. 4). Micrographs of whole seedlings in Figure 3A were assembled from individual pictures using Adobe Photoshop Creative Suite 4. The lipophilic dye FM4-64 (Molecular Probes) was used at a concentration of 10 μg mL^{-1} to demarcate cell membranes in Figure 4, B to D. Imaging for Figure 4, B and C was done using a Zeiss 510 Meta laser scanning confocal microscope with a 63× air-water dipping lens using the multitracking mode.

Transient Expression in Protoplasts

Protoplast isolation and transfection experiments were performed as reported (Sheen, 1990; Yoo et al., 2007). All protoplast experiments were performed in duplicates, and independent biological replicates yielded similar results.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Summary of 5′-(A/G)GAT(T/C)-3′ hits in cytokinin target genes, control genes, and random sequence.

Supplemental Table S2. List of clustered 5′-NNN(A/G)GAT(T/C)NNN-3′ hits in cytokinin target genes and control genes.

Supplemental Table S3. Distance and orientation of clustered hits in cytokinin target genes and control genes.

Supplemental Table S4. Sites used for *TCSn*.

Supplemental Table S5. Plasmid and oligonucleotide sequences.

Supplemental Table S6. Gene sequences used for the bioinformatic analyses.

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3 Results PUP14

Characterization of *PUP14* functions in plant development

The *TCSn* reporter has allowed us to determine cytokinin signalling patterns *in planta* unravelling that signalling occurs in defined areas. It is however not clear how these distinct locales are specified. In this chapter, we report that PUP14 modulates the distribution of active cytokinins and thereby defines cytokinin signalling landscapes.

All of Chapter 3.1 is a manuscript draft.

Author Contributions

B.M. devised experiments, constructed the PUP14 reporter, inducible AHK3 and CKI1 constructs and the expression plasmids, did microscopic imaging and wrote the manuscript, E.Z. constructed all other constructs, generated transgenic plants, performed crosses, did transport experiments with protoplasts and seedlings, performed qRT-PCR analyses, and wrote the manuscript. J.L. performed the protoplast reporter assays, M.D. and M.G. performed transport experiments with microsomes. All authors contributed to the writing of the manuscript.

Please find the Extended Data attached as Appendix A1.

3.1 CYTOKININ IMPORTER PURINE PERMEASE 14 CONFINES THE CYTOKININ RESPONSES

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ABSTRACT

Multicellular organisms operate via the coordination of specialized cellular identities and functions dictated by signalling systems. However, the mechanisms that define the precise spatiotemporal cell domains and subcellular locales responding to specific signals are not well understood. In plants, cytokinins direct essential cell-to-cell signalling systems to initiate and maintain differential cell functions during gametophyte development, embryogenesis and postembryonic shoot and root development (Hwang et al., 2012; Kieber and Schaller, 2014). Guided by the live green fluorescent protein (GFP) imaging of the synthetic reporter, TCSn::GFP (Two Component signalling Sensor) (Zürcher et al., 2013), we illuminate precise cell populations activating conserved phosphorelay signalling circuitry despite much broader intracellular cytokinin signalling competence and potential. Here, we report a surprising finding that Arabidopsis *PURINE PERMEASE 14* (*PUP14*) plays a pivotal role in confining the cytokinin signalling response throughout development by ligand sequestration. The expression patterns of *PUP14* are inversely correlated with the cytokinin signalling readout, indicating an antagonistic relation. Indeed, the conditional *PUP14* knock-down by an artificial microRNA (*amiRPUP14*) causes ectopic cytokinin signalling accompanied by aberrant morphogenesis in embryos, roots and the shoot apical meristem. PUP14 protein localizes to the plasma membrane and imports bioactive cytokinins as shown by analysing transport in mesophyll protoplasts, seedlings and microsomes. These activities of PUP14 reduce the apoplastic cytokinin pools and extracellular cytokinin perception by cytokinin sensors. The relevance of apoplastic cytokinins in initiating intracellular signalling is corroborated by our findings that signalling output is attenuated by secreted cytokinin-degrading enzyme CYTOKININ OXIDASE 2 (CKX2) (Werner et al., 2003), but not by intracellular CKX variants. These results uncover the existence of a previously unknown and dedicated transport system that patterns the cytokinin signalling landscape of vascular plants.

RESULTS AND DISCUSSION

Chemical hormones including cytokinins are key signals to govern pattern formation and morphogenesis throughout the plant life cycle. However, the mechanisms that define the precise spatio-temporal domains of cytokinin perception (Zürcher et al., 2013) are largely unknown. To address the question of how the localized signalling activities are established, we used heart-stage embryos as a model where the cytokinin response marks the provascular tissue (Fig. 1a). Formally, both the availability of bioactive cytokinins and the cellular competence to respond to a stimulus may control the signalling domains. To determine whether active cytokinins are limiting, heart-stage embryos were incubated for 16 h with an excess of the degradation-insensitive cytokinin benzyl adenine (BA) (Galuszka et al., 2007). This caused a stereotypical expansion of the *TCSn::GFP* response domain (Zürcher et al., 2013) (Fig. 1b). However, no *TCSn::GFP* induction was observed in the prospective cotyledons, despite the transcription of the cognate cytokinin receptor *ARABIDOPSIS HISTIDINE KINASE 4 (AHK4)* in these domains (Mähönen et al., 2000) (arrowheads Fig. 1c). Ethanol-induced expression (Roslan et al., 2001) of an *AHK3* transgene encoding a redundantly acting cytokinin receptor (*35S>ALC>AHK3*) together with an excess of BA did not activate *TCSn::GFP* in prospective cotyledons either (arrowheads Fig. 1d), supporting the notion that failure to turn on signalling is not due to absence of the receptors. To test whether signalling downstream of receptors is functional, we expressed *CYTOKININ INDEPENDENT 1 (CKI1)* for 16 h using the ethanol-inducible system (*35S>ALC>CKI1*). *CKI1* encodes a hybrid kinase with cytokinin-independent constitutive activity (Hwang et al., 2012) and caused ubiquitous *TCSn::GFP* activation (Fig. 1e). Together, these results suggest that cells of the prospective cotyledons fail to activate cytokinin signalling despite abundant active ligands and a functional signalling system. We hypothesized that productive ligand-receptor interactions *in planta* depend on cytokinin transporters that guide differential cellular localization of cytokinins. However, the genes that fulfil this postulated role have not been identified. Members of the vascular plant-specific family of transmembrane PURINE PERMEASES (PUPs) have been implicated in cytokinin translocation (Gillissen et al., 2000); yet, a link to cytokinin function *in planta* was not established. To test whether uncharacterized members of the *Arabidopsis PUP* family control the spatio-temporal landscape of cytokinin signalling, we first established a transcription profile of the family members based on our own analysis and published transcriptome data (Belmonte et al., 2013; Yadav et al., 2014; Adrian et al., 2015) (Extended Data Fig. 1). *PUP14* expression prevailed in all organs and stages analysed, including embryos. To determine the *PUP14* expression pattern, we analysed *PUP14::PUP14-GFP* transgenic plants. In heart-stage embryos, PUP14-GFP localized to cells of the prospective cotyledons (Fig. 1f) that failed to activate cytokinin signalling (Fig. 1b). We found *PUP14* expression in the seedling's main root (Fig. 1i), the lateral root primordia (LRP) (Fig. 1l), the shoot apical meristem (Fig. 2a), and in ovules and seeds (Extended Data Fig. 3a-b) exhibiting equivalent complementary patterns to those of cytokinin signalling (Fig. 1j,m, 2b and Extended Data Fig. 3a,b).

The near-exclusive nature of *PUP14* expression and cytokinin signalling patterns is compatible with an inhibitory function of PUP14 in the cytokinin response. To test this hypothesis, we constructed an ethanol-inducible artificial microRNA (Schwab et al., 2006) targeting *PUP14 (35S>ALC>amiRPUP14)*. Upon induction of the transgene, *PUP14* mRNA

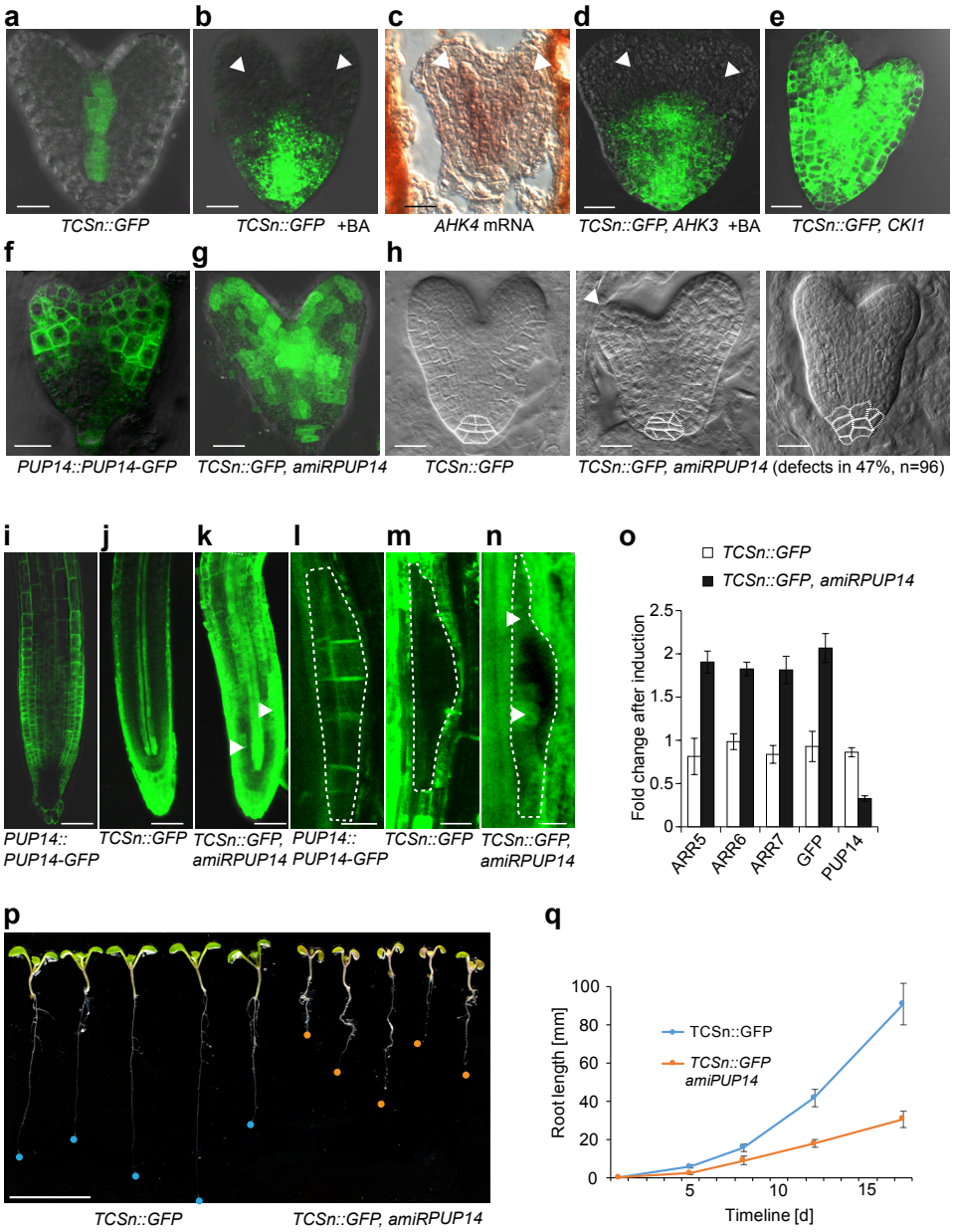

Figure 1 | PUP14 confines the cytokinin response in embryos and roots
a,b,d,e, Micrographs of *TCSn::GFP* in heart-stage embryos overlaid with transmitted light pictures subjected to 16 h (a) mock, (b,d) BA treatments, (d)16 h-induced *AHK3* expression, or (e) CKI1 (see methods), arrowheads denote prospective cotyledons without *TCSn::GFP* expression. c, *AHK4* mRNA detected by in situ hybridisation. f, PUP14::PUP14-GFP in heart stage embryo. g, Ectopic TCSn::GFP 16 h after *amiRPUP14* induction (85% of embryos, n=53). h, Morphological defects 48h after *amiRPUP14* induction (47% of embryos, n=96) including reduced size of prospective cotyledons (arrowhead) and cell division defects in the root meristem (cell boundaries outlined with white dotted lines). i, PUP14::PUP14-GFP in main root. j, TCSn::GFP in the main root. k, Ectopic cytokinin responses after *amiRPUP14* induction (arrowheads). l, PUP14::PUP14-GFP in LRP (denoted by dotted line). m, TCSn::GFP in LRP. n, Ectopic TCSn::GFP in *amiRPUP14* induced LRP (arrowheads). o, Relative changes of type-*A ARR5*, *6* and *7*, and *TCSn::GFP* (as a group significantly different: $p<0.001$ from unpaired t-test, *TCSn::GFP*: n=4; *TCSn::GFP, amiRPUP14*: n=4) and *PUP14* mRNA levels (significantly different: $p<0.001$ from unpaired t-test, *TCSn::GFP*: n=4; *TCSn::GFP, amiRPUP14*: n=7) after 16 h of *amiRPUP14* induction in 7 d old seedlings of indicated genotype, assessed by quantitated real-time (qRT)-PCR, error bars represent s.e.m. p, Seedlings after 7 d on ethanol-containing medium. q, Growth curve of seedlings shown in (p). Scale bars (a-h) 20 µm, (i-k) 50 µm, (l-n) 10 µm, (p) 1 cm, +BA, 16 h treatment with 10µm BA.

and PUP14-GFP levels were strongly reduced within 24 hrs of induction (Extended Data Fig. 2a-c), demonstrating the efficacy of this approach. The phenotypes of *amiRPUP14*-induced embryos and seedlings were complemented by an *amiRPUP14*-resistant transgene *(PUP14*)* encompassing the *PUP14* locus (Extended Data Fig. 2c-e), validating that the inducible *amiRPUP14* acts specifically. Inducing *amiRPUP14* expression for 16 h caused widespread ectopic cytokinin signalling in the embryo (Fig. 1g), also in cells of the prospective cotyledons that are non-responsive to treatments with exogenous cytokinins (arrowheads Fig. 1b), supporting the role of PUP14 in confining the cytokinin response. The same treatment did not affect the auxin response (Extended Data Fig. 2f), indicating that PUP14 acts specifically on cytokinin signalling.

Longer inductions caused morphological defects in prospective cotyledons and the nascent root meristem (Fig. 1h). Similarly, *amiRPUP14* induction resulted in ectopic cytokinin signalling in the seedling root, particularly in the meristematic region of the root tip (Fig. 1k)

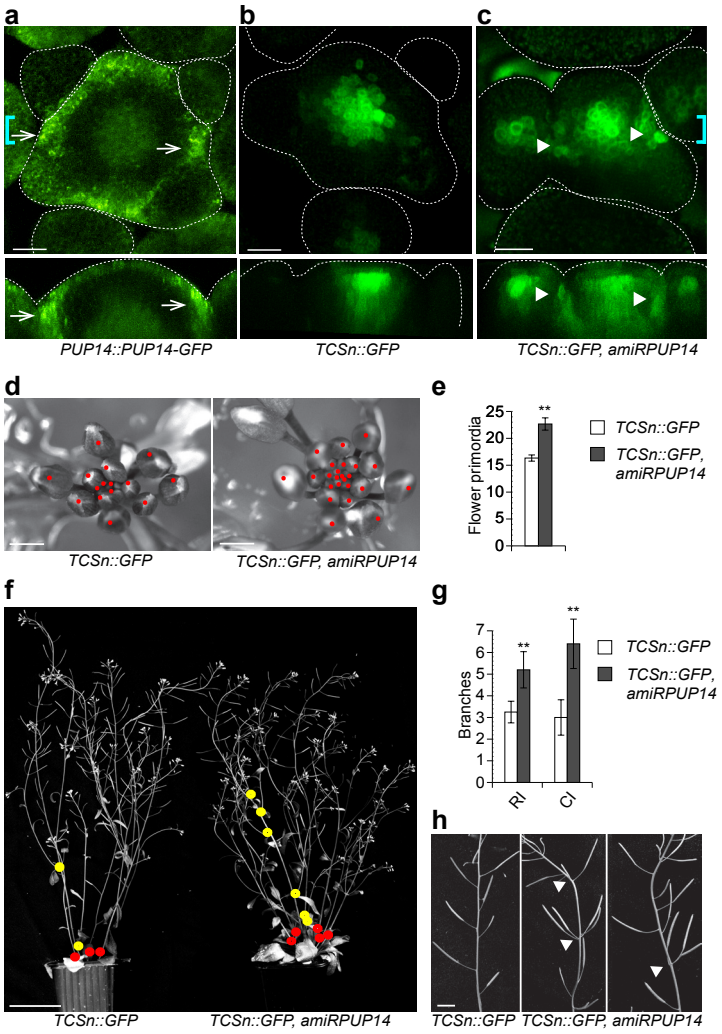

Figure 2 | PUP14 confines the cytokinin response in the SAM
a, PUP14::PUP14-GFP, and b, TCSn::GFP in floral SAM. Arrows indicate peak PUP14-GFP levels at organ-organ boundaries. c, Ectopic TCSn::GFP (arrowheads) after *amiRPUP14* induction. Longitudinal optical sections in lower panels (a-c) at cyan-coloured brackets, dotted lines mark organ boundaries. d-h, Comparisons of ethanol-treated *TCSn::GFP*, and *TCSn::GFP, amiRPUP14* phenotypes. d, Inflorescences. e, Numbers of flower primordia at stages 6-12 on the main apex (Smyth et al., 1990), n=6. f, Shoot architecture. Red dots denote primary rosette branching (RI), yellow dots primary cauline branching (CI). g, Number of RI and CI, n=6. h, Inflorescence stems, arrowheads indicate perturbations. Data represent mean value, error bars represent s.d. **p < 0.01 unpaired t-test. Scale bars (a-c) 20 µm, (d) 1 mm, (f) 5 cm, (h) 1 cm.

and LRP (Fig. 1n). Accordingly, transcription of the immediate-early cytokinin target genes type-A *ARABIDOPSIS RESPONSE REGULATORS (ARR) ARR5*, *6* and *7* (D'Agostino et al., 2000) was induced in seedlings (Fig. 1o). Continuous induction of *amiRPUP14* led to a strong growth retardation of the seedling root, abnormal cotyledons, and a suppression of lateral roots (Fig. 1p,q), consistent with increased cytokinin activities (To et al., 2004). In the shoot, cytokinin controls the homeostasis of the shoot apical meristem (SAM)(Gordon et al., 2009), where increased cytokinin causes a more active meristem with more primordial (Bartrina et al., 2011). Reduction of *PUP14* levels after *amiRPUP14* induction caused ectopic cytokinin output in the SAM (Fig. 2c), which was accompanied by a higher number of primordia (Fig. 2d,e), increased shoot branching and disturbed phyllotaxis (Fig. 2f-h).

Similar phenotypes have been observed in plants mutant for *CYTOKININ OXIDASE (CKX) 3* and *5* (Bartrina et al., 2011), *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (Besnard et al., 2014), and *ARR3-9* (Leibfried et al., 2005), which also display ectopic cytokinin activities. Together, these findings strongly support that PUP14 functions to limit the cytokinin response domains throughout development.

Next, we addressed the cellular function of PUP14. PUP14-GFP fusion proteins localize to the plasma membrane (Fig. 1f,l). To test PUP14's cytokinin transport capacity, we conducted uptake experiments using labelled trans-zeatin (tZ), an abundant natural cytokinin (Werner et al., 2003). Transient expression of *PUP14* in mesophyll protoplasts or tobacco microsomes stimulated the uptake of labelled tZ (Fig. 3a,b). The PUP14 transport activity was ATP-dependent and higher compared to PUP1 (Gillissen et al., 2000) (Fig. 3b). Uptake was inhibited by unlabelled tZ, by the common natural cytokinin isopentenyl adenine (iP), by the aromatic cytokinin BA, and also by adenine, but not by tZ riboside, the major cytokinin transport form (Beveridge et al., 1997), nor auxin (IAA), nor allantoin, which is an unrelated substrate (Fig. 3c,d). Energy-dependent cytokinin uptake into a microsomal cell-free system further excludes that uptake is dependent on cytoplasmic metabolisation. Conversely, seedlings with decreased *PUP14* levels exhibited a reduced uptake rate for exogenously added tZ compared to control seedlings (Fig. 3e).

Our data show that plasma membrane localized PUP14 imports bioactive cytokinins, implying that PUP14 activity depletes ligands from the apoplast, which leads to a suppression of the cytokinin response. In this scenario, extracellular cytokinins binding to the sensing domains of plasma-membrane localized receptors (Kim et al., 2006; Wulfetange et al., 2011) (Extended Data Fig. 3c,d) are important to initiate the signalling response, while intracellular cytokinins binding to endoplasmic reticulum (ER)-localized receptors (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011) would contribute less for activation of the pathway. To test this hypothesis, we devised experiments that compare the effects of differentially targeted cytokinin-degrading enzymes on the cytokinin signalling response. Mesophyll protoplast cells responded to as little as 100 pM of exogenously added tZ by activating cytokinin signalling (Müller and Sheen, 2008), suggesting they depend on exogenous cytokinins, and thus serve as a suitable model to study cytokinin perception independent of production (Fig. 3f). Transient transfection with plasma membrane-localized

PUP14 (Fig. 3h) caused a marked reduction of cytokinin-dependent *TCS::LUCIFERASE (LUC)* activity, in agreement with loss of *PUP14* function that caused ectopic cytokinin responses (Fig. 1g,k,n,o, 2c). Similarly, transient expression of a wild-type CKX2 that is targeted for secretion to the apoplast (Werner et al., 2003) (Fig. 3i) attenuated the cytokinin response. In contrast, a variant of CKX2 that lacks the N-terminal signal peptide (CKX2ΔSP) and localizes to the cytoplasm (Fig. 3j) did not affect the cytokinin response. Neither did CKX7, which has been reported to localize to the cytoplasm (Köllmer et al., 2014) (Fig. 3k), Finally, a CKX2 variant that is targeted to the lumen of the ER (CKX2-ER) (Fig. 3l) did not significantly reduce the signalling response either (Fig. 3f). The outcomes of these experiments support the dominant role of apoplastic cytokinins to trigger signalling in target cells via plasma membrane-localized AHK receptors (Kim et al., 2006; Wulfetange et al., 2011) (Extended Data Fig. 3c,d).

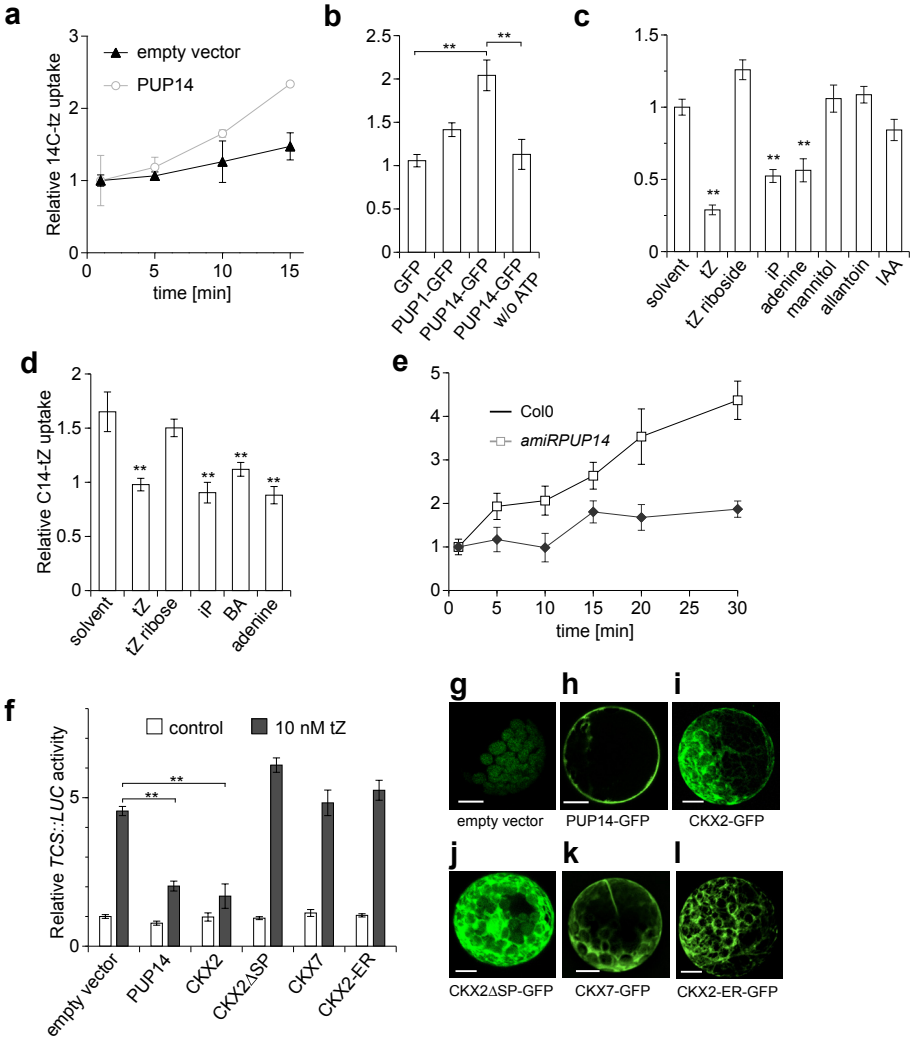

Figure 3 | PUP14 cellular function
a-e, Relative ^{14}C-tZ uptake rates on y-axis. a, Transfected mesophyll protoplasts. b, Transport in microsomes derived from *35S::GFP*, *35S::PUP1*, or *35S::PUP14* transfected N. benthamiana. c, Competition by indicated substances in *PUP14*-transfected protoplasts. d, Competition in microsomes of *35S::PUP14* transfected *N. benthamiana*. e, *amiRPUP14* vs. Col-0 seedlings. f, Relative TCSn::LUC activities with 10 nM tZ in mesophyll protoplasts co-transfected with transgenes as indicated, normalised to empty vector control. g-l, Subcellular localization of transfected gene products as shown in (f). Data represent mean value, error bars represent s.d. (a) or s.e.m. (b,c,d,e,f), **p < 0.01, ANOVA with Tukey's HSD post hoc test. Scale bars 10 µm.

Based on our findings, we propose a model (**Extended Data Fig. 4**) where PUP14 activity inversely correlates with the capacity of a cell to sense cytokinins. PUP14's cytokinin import activity leads to a translocation of cytokinins from the apoplast to the cytosol, away from sensing domains of plasma membrane-localized receptors, which causes a reduction in cytokinin signalling. Feeding experiments with radiolabeled bioactive cytokinin suggested that the bulk of imported cytokinins are inactivated by conversion to monophosphates by ADENINE PHOSPHORIBOSYL TRANSFERASE enzymes (Moffatt et al., 1991; Zhang et al., 2013). Furthermore, *N*- or *O*-glycosylation, oxidative cleavage, or transport to other cells may contribute to clearance of intracellular cytokinins (Kieber and Schaller, 2014). In addition to PUP14, other members of the family are likely to control cytokinin signalling in defined developmental contexts. The fact that *PUP* genes are specific to vascular plants (Hildreth et al., 2011) may suggest their function to support more complex cytokinin signalling patterns associated with the bauplan of land plants.

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MATERIALS AND METHODS

Microscopy and live imaging

RNA in situ hybridization and embryo clearings were observed with a transmission microscope under bright field or differential interference contrast with a 40x oil immersion lens. For GFP imaging, live embryos or seedlings were mounted in 0.5 strength Murashige and Skoog (MS) dissolved in water. SAMs were mounted in warm 0.8% low-gelling agar dissolved in 0.5 strength liquid MS. Confocal microscope observations were done on a SP2 or SP5 spectral detection confocal microscope (Leica) equipped with a 20x glycerol immersion (seedling root, seeds), 40x oil immersion (SAM) or 63 glycerol immersion lens (female gametophytes, embryos). Images were processed using Imaris (Bitplane, Zurich). Maximum partial projections of equivalent serial sections are shown. Adult plants were recorded with a DP3 Merrill digital camera (Sigma), and flower primordia with a MZFLII fluorescence stereomicroscope equipped with a DFC 420C digital camera (Leica).

Plant material and growth conditions

The ecotype Col-0 was used as wild type. Seeds were surface sterilized for 18 min in 5 % (v/v) bleach and 0.1 % (v/v) triton-X, washed three times in sterile ddH_2O and kept in the dark at 4 °C for a minimum of 2 days for stratification. Seeds were sown on 0.5 strength MS medium with 2 % (w/v) sucrose, 0.8 % (w/v) phytagar and 2 mM MES pH 5.6 containing the appropriate antibiotic or herbicide for selection. Seeds on selection plates were placed into a Percival plant incubator (CU-36L6/D Percival Scientific Inc., Perry IA, USA) with 22°C and a 14/10 h light/dark regime with 120 mmol m^{-2} s^{-1}. To phenotype seedlings, plants were grown vertically on 12 cm square plates containing 10 mL of medium. To prevent desiccation of the plates, 1 mL of ddH_2O was added and the plates were sealed with parafilm. Seeds on vertical plates were placed into a Percival plant incubator (CU-36L6 Percival Scientific Inc., Perry IA, USA) with 22°C and 12/12 h light/dark cycles with 90 mmol m^{-2} s^{-1}. For long-term growth, seedlings were transferred to soil and grown at 22 °C during the day, 20 °C at night with a 16/8 h photoperiod.

Plant transformation

Plant transformation was performed using *Agrobacterium tumefaciens* of the GV3101 strain (Clough and Bent, 1998). Inducible expression constructs were super transformed into *TCSn::GFP* or *PUP14::PUP14-GFP* transgenic lines.

Ethanol induction of transgenes

Ethanol was applied as follows to induce expression of *amiRPUP14*, *CKI1*, or *AHK3* from the ethanol-inducible two-component system (Roslan et al., 2001). For phenotype assessments of seedlings, 1 mL of 1 % (v/v) ethanol was added to the bottom of the vertical plates 4 days after germination. For expression analyses, 7 day old seedlings grown on vertical plates were transferred to 6-well culture dishes with 3 mL of liquid medium (0.5-strength MS, 2 % (w/v) sucrose, 2 mM MES pH 5.7) with and without 1 % (v/v) ethanol) for 16 - 24 h.

Dishes were sealed with parafilm. Induction in adult plants was by watering with 1 % (v/v) ethanol every 4 days starting from bolting stage as described (Roslan et al., 2001). Embryo inductions were performed as described (Müller and Sheen, 2008). Controls shown are *TCSn::GFP* treated with ethanol in parallel to the experimental genotypes. Similar results were obtained with untreated *TCSn::GFP, amiRPUP14* plants (see Fig 1o).

Constructs

For *PUP14::PUP14-GFP*, *PUP14* encompassing the locus including the 2.3 kb upstream fragment was amplified from Col0 genomic DNA by PCR and cloned into the binary vector pCB302(Xiang et al., 1999) with the enhanced GFP coding sequence for C-terminal fusion, nopaline synthase 3' untranslated region and ligation-independent cloning (LIC) (Aslanidis and de Jong, 1990) adaptors. For protoplast studies, *PUP14, CKX2, CKX7, AHK4* genomic regions from translational start to stop were amplified from Col0 genomic DNA and annealed to LIC-modified expression vectors to yield 2HA or GFP C-terminal translational fusions. The artificial microRNA (amiR) constructs were designed with the Web MicroRNA Designer (www.weigelworld.org), assembled by PCR amplification on the pRS300 as template (Schwab et al., 2006). Sequences of the *amiRPUP14_1* and *amiRPUP14_2* are TTATTTGCACAAAGTGTTCTG and TGTTGATAGGTATTTGCACGA, respectively. Both *amiRPUP14* constructs caused similar phenotypes upon induction. Corresponding target sites in PUP14 are CAGAACAATTTGTGCAAATAC and TTGTGCAAATACCTATCAACA. The PCR amplicons were cloned into the LIC-modified DM7 vector (Zürcher et al., 2013). amiR-resistant versions of *PUP14 (PUP14*)* were constructed by site-directed mutagenesis of the *amiRPUP14_2* target site to change all codons within the amiR target site to synonymous codons with overall comparable codon usage frequency. The PUP14* encompasses the *PUP14* genomic region and was cloned into pCB302 by LIC. 35S>ALC>AHK3 cloned by LIC with PCR-amplified insert from Col0 genomic DNA. *35S::PUP1* and *35S::PUP14* for expression in microsomes were amplified from Col0 genomic DNA, and cloned into pPLV26 (De Rybel et al., 2011) by LIC. All constructs were sequenced to ensure no unwanted mutations were introduced. A plasmid list is provided in Extended Data Table 2.

Protoplast isolation and transfection

For transient expression experiments, protoplasts of three- to four-week-old wild-type *Arabidopsis* plants of the Col0 ecotype were isolated as described (Yoo et al., 2007) with the following adaptations: adjusted concentration was 3 x 10^5 ml^{-1}, *35S::renillaLUC* was used to normalise for transfection efficiency (Bielach et al., 2012) of *TCS::LUC* reporter assays, and W1 solution was supplemented with 15 mM sucrose. For reporter assays, transfected protoplasts were incubated over night, tZ or solvent at indicated concentrations were added, and protoplasts were harvested 90 min later for LUC measurements. For transport assays, transfections were scaled up according to needs and purified plasmids were transfected in 1:1 ratio between effector and empty plasmid. Transfected protoplasts were cultivated between 12 and 24 hrs at 22 °C in light (120 mmol m^{-2} s^{-1}). Means and standard error of means of at least three independent experiments with three technical replications each are represented.

Transport assays

For protoplast transport assays, protoplasts were harvested at 100 rcf for 2 min and resuspended in percoll solution (0.5 M Sorbitol, 1mM $CaCl_2$, 20 mM MES NaOH pH 5.6, 25 % (v/v) percoll) and mixed with the same volume of glycine betaine solution (0.5 M glycine betaine, 1 mM $CaCl_2$, 20 mM MES NaOH pH 5.6) containing ^{14}C- labelled tZ and $^{3}H_2O$. The final concentration of labelled tZ was 1 or 2 µM. For competition studies, unlabelled cold substrate was added in a 100- fold excess. Transport was stopped by centrifugation of samples on a percoll cushion after indicated time points. For scintillation counting, pelleted protoplasts were transferred into 3 mL of Ultima Gold™ (PerkinElmer AG, Schwerzenbach, Switzerland) and subjected to 10 min of disintegration counting of ^{14}C and ^{3}H. Three independent replicates of the uptake experiment were conducted with similar results, and means with standard deviations from one representative experiment with four technical replications are shown. For competition assays, mean values from three independent experiments with each four technical replications are shown. Indicated relative uptake was calculated as the radioactivity of ^{14}C per radioactivity of $^{3}H_2O$ normalized to the first time point (30 s). For seedling transport assays, twelve-day old induced seedlings were transferred to 0.5 x MS, 2 % (w/v) sucrose, 2 mM MES and vacuum infiltrated for 5 min and twice 3 min. For each replicate >10 mg of plant material was used. Radiolabelled tZ was added to a final concentration of 2 µM in 2 mL. Seedlings were washed after indicated time points with excess volumes of cold 0.5 MS, 2 % (w/v) sucrose, 2 mM MES on a Büchner funnel. Seedlings were dried on filter paper and transferred to 1.5 ml tubes containing 800 mL of 80 % (v/v) ethanol and heated for 5 min at 95 °C. Samples were transferred into scintillation vials containing 3 mL Ultima Gold™ (PerkinElmer) and subjected to 2 min disintegration counting of ^{14}C. Indicated relative uptake was calculated as the radioactivity per fresh weight normalized to the radioactivity per fresh weight at the first time point (1 min). Mean values from 3 independent experiments with each 4 technical replications are shown. For microsomal uptake experiments, *35S::PUP1*, *35S::PUP14* and *35S::GFP* were transiently expressed in *N. benthamiana* leaf tissue by *Agrobacterium tumefaciens*-mediated transfection and microsomes were prepared as described (Henrichs et al., 2012). For tZ-uptake experiments, ^{14}C-labelled tZ was diluted into transport buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 % sucrose, pH 7.6 with or without 5 mM ATP) and added to 300 µg of microsomes to yield a final concentration of 1µM labelled tZ. For substrate competition assays unlabelled substrate was included in the transport buffer at a 100-fold excess. After 10 s and 4 min of incubation at 20°C, aliquots of 100 µL were vacuum-filtered on Whatman™ NC45 filters (GE Healthcare, Little Chalfont, UK) and washed 3 times with 1 mL cold ddH_2O. Air-dried filters were objected to scintillation counting as described below. Indicated relative uptake was calculated as the radioactivity normalized to the first time point (10 s). Means and standard error of means of at least four independent experiments with three technical replications each are represented.

qRT PCR analysis

Quantification of relative gene expression was done by qRT-PCR on an Applied Biosystems 7500 Fast Real- Time PCR System using SYBR® Green PCR Master Mix (Applied

Biosystems, Life Technologies Europe B.V., Zug, Switzerland) or SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories AG, 1785 Cressier, Switzerland) according to manufacturer's recommendation. Final primer concentrations were 400nM in a total volume of 20µl. The relative values of the transcripts were normalized to *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (eIF4A, At3G13920) levels. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Means and standard error of means of at least four independent experiments with three technical replications each are represented.

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The following experiments are not included in the manuscript but will still be presented in this thesis.

3.2 FURTHER RESULTS FOR PUP14

RESULTS

Testing of amiR efficacy by ETPamir screening

PUP14 knockdown mutants were generated using amiR-mediated downregulation of *PUP14*. In order to determine the *in vivo* efficacy of the best-ranked *amiR* candidates obtained through the use of the WMD web designer (http://wmd.weigelworld.org), I performed an epitope-tagged protein-based amiRNA (ETPamir) screen (Li et al., 2013, 2014). Isolated protoplasts were transiently co-transfected with HA-tagged PUP14 (*PUP14-HA,* target gene) and the three best-ranked amiRs, *amiR14_1*, *amiR14_2*, *amiR14_3*, to be tested. *PUP4-GFP* was used as a control gene to test the specificity of the amiRs.

Already after 18 hrs of induction, a reduction of PUP14-HA protein could be detected for protoplast samples transformed with any of the 3 *amiR* candidates compared to protoplasts that were transfected without an *amiR* (Fig. 1a). For *amiR14_1* and *amiR14_2*, a further decrease in signal intensity could be observed after 24 hrs, whereas the detected PUP14-HA band in samples transfected with *amiR14_3* is comparable to the one detected after 18 hrs. The PUP14-HA levels for *amiR14_2* transfected protoplasts remain at a low level within the 4 points in time assessed (18, 24, 38, 48 hrs). Samples transfected with *amiR14_1* show recurring PUP14-HA signal at 48 hrs. Levels after 38 hrs incubation cannot be evaluated due to a white stain at the level of the apparent PUP14-HA band. The expected size for PUP14-HA is 46.24 kDa but for unknown reasons, a double band is detected. As controls, the detection of PUP4-GFP was run in parallel (Fig. 1b). The tagged protein has a calculated mass of 69.22 kDa. A faint band at around 70 kDa can be distinguished at 38 and 48 hrs of incubation. At all other assessed incubation times, no band of that size is evident; instead, no bands or several bands with varying sizes are apparent.

The observed patterns indicate that the tested *amiR* construct negatively affect the levels of PUP14-HA with *amiR14_2* displaying the most persistent downregulation, and *amiR14_1* showing a slightly weaker level of downregulation. However, the lack of a detectable, consistent signal for the control, PUP4-GFP, prevents us from drawing any conclusion in regards to the specificity of the tested *amiR* constructs.

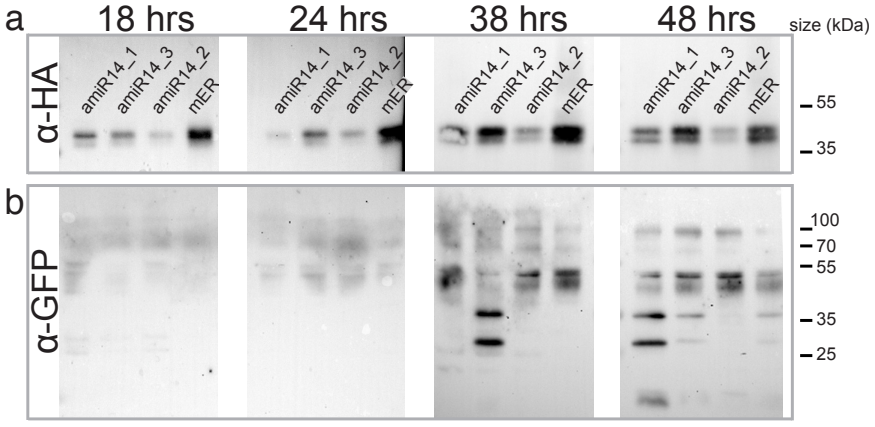

Fig. 1 | ETPamiR identifies *amiR14_2* as efficient construct for PUP14 downregulation.
a) PUP14-HA levels are detected after indicated *amiR* induction periods. *amiR14_2* causes a consistent reduction of PUP14-HA levels. b) The control blots for PUP4-GFP did not yield interpretable results.

Effects of PUP14 *overexpression on* TCSn::GFP *signalling*

As we have shown in the previous chapter, the specific localization of signalling output can be dissipated by the induced downregulation of *PUP14*. To visualize the effects of *PUP14* deregulation on cytokinin signalling *in vivo*, I transformed *TCSn::GFP* plants with an alcohol inducible *35S>Alc>PUP14* (*PUP14OX*) transgene. TCSn::GFP expression was analysed in embryos from siliques of *PUP14OX* plants after ethanol treatment. After 24 hrs of induction with 1 % (v/v) ethanol, embryos showed varying effects on cytokinin signalling (Fig. 2). In 18 % (10/57) of ethanol-treated embryos, cytokinin signalling appeared not to be affected (Fig. 2a). Mock-treated samples showed normal signalling output in 42 % (8/19) of cases. In 33 % (19/57) of the analysed ethanol-treated embryos, ectopic TCSn::GFP signal was observed with a simultaneous weakening of the endogenous pattern (Fig. 2b). In 30 % (17/57), only a weak TCSn::GFP signal was detected but in the endogenous pattern (Fig. 2c). The numbers of induced samples with dampened TCSn::GFP signal are comparable to the 32 % (6/19) mock-treated embryos displaying weak, but normally localized signal. Nineteen per-cent (11/57) of ethanol-treated compared to 26 % (5/19) of mock-treated embryos did not show TCSn::GFP (Fig. 2d). In mock-treated embryos ectopic signalling was never observed. TCSn::GFP transgenic embryos without the overexpression construct that were assessed in parallel showed strong provasculature signal in 67 % (4/6) and weaker signal in 33 % (2/6) of cases. These observations indicate that the overexpression of *PUP14* causes variable alterations in the embryonic cytokinin signalling landscape, which is noticed primarily by changes in signalling distribution.

Validation of PUP14 overexpression

Due to the varying effects on embryonic cytokinin signalling observed with the inducible *PUP14* overexpression, we chose to assess the efficiency of induction by recording PUP14-GFP levels using a *35S>Alc>PUP14-GFP* transgene (*PUP14-GFP OX*). We analysed embryos after 24 hrs of incubation in 0, 0.01, 0.1 or 1 % (v/v) ethanol-containing medium (Fig. 2e-j). The transgene was not induced at detectable levels by 0 or 0.01 % ethanol (Fig. 2e, f). We detected GFP signal in embryos incubated in concentrations of 0.1 % ethanol or beyond. Induction with 0.1 % ethanol caused PUP14-GFP expression in discrete cells, primarily in the root apical meristem or uppermost suspensor cells (Fig. 2g), whereas induction with 1 % ethanol yielded detectable PUP14-GFP signal in the in the root apical meristem (Fig. 2h), uppermost cells of the suspensor, in cells of the future cotyledons (Fig. 2h), and on the surface of the embryo (Fig. 2i). The pattern of PUP14-GFP expression remained patchy also in embryos treated with 1 % ethanol and the degree of induction varied within and between the two lines analysed. These results show that PUP14-GFP is not evenly induced by ethanol treatment in embryos. Because the system was reported to cause uniform expression (Roslan et al., 2001), the findings suggest that posttranscriptional regulatory mechanisms operate to manage PUP14 levels *in vivo*.

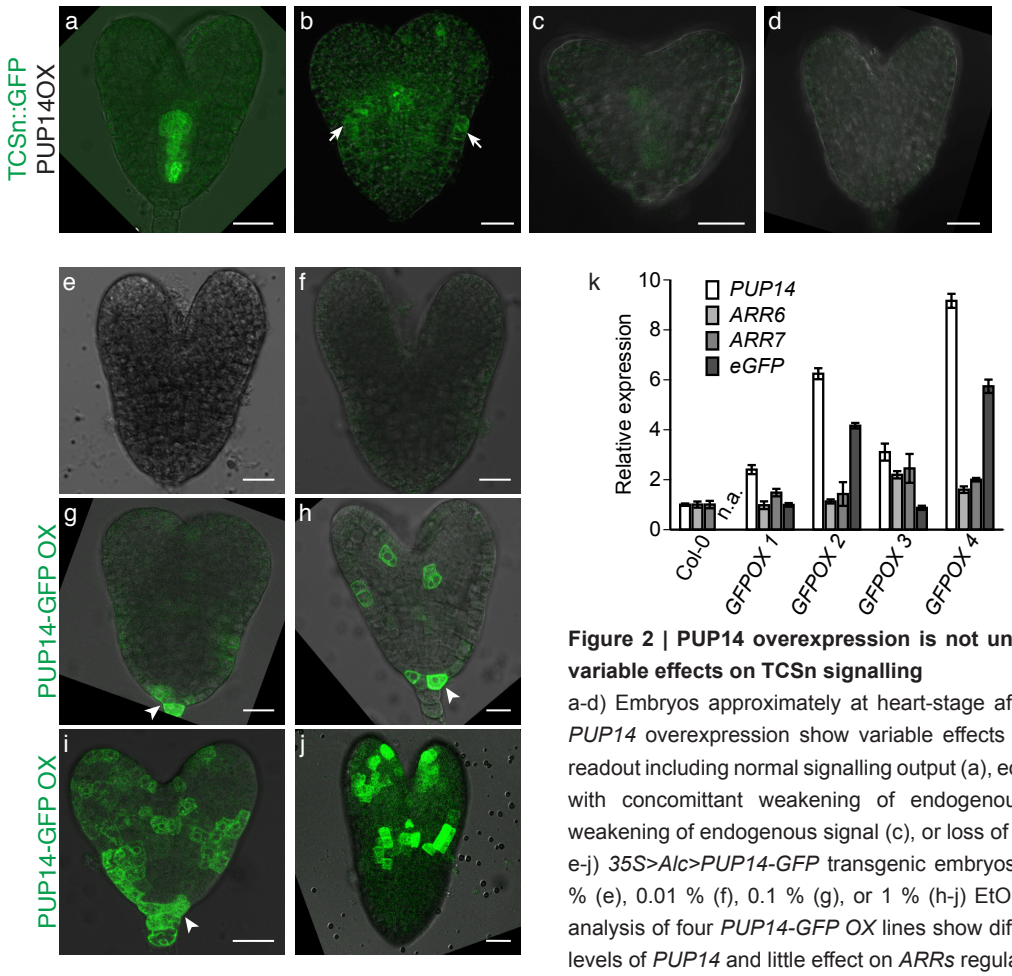

Figure 2 | PUP14 overexpression is not uniform and has variable effects on TCSn signalling

a-d) Embryos approximately at heart-stage after induction of *PUP14* overexpression show variable effects on *TCSn::GFP* readout including normal signalling output (a), ectopic signalling with concomittant weakening of endogenous pattern (b), weakening of endogenous signal (c), or loss of signalling (d). e-j) *35S>Alc>PUP14-GFP* transgenic embryos treated with 0 % (e), 0.01 % (f), 0.1 % (g), or 1 % (h-j) EtOH. k) qRT-PCR analysis of four *PUP14-GFP OX* lines show different induction levels of *PUP14* and little effect on *ARRs* regulation.

Scale bars = 20 µm; arrows indicate ectopic signalling; arrowheads indicate strong PUP14-GFP localization in suspensor and/or root apical meristem cells. Values in k) are mean ± SD of 3 technical replicates of a single experiment.

Expression analysis in seedlings of PUP14-GFP

To address the question of whether the transgene is principally induced, *PUP14-GFP* induction of ethanol-treated *PUP14-GFP OX* seedlings was assessed by qRT-PCR. The expression profile of the four studied lines reveal 2.4 – 9.2 x higher total *PUP14* transcript levels compared to the tested Col-0 wild type (Fig. 2k). As the utilized *PUP14* primers do not distinguish between endogenous *PUP14* and *PUP14-GFP*, I specifically assessed the *PUP14* transgene by measuring *GFP* transcripts. Since the wild-type Col-0 does not express *GFP*, these measurements only reflect relative levels within each sample and do not portend the induction compared to the wild-type. The *GFP* levels in the transgenic lines are positively correlated with the detected levels of total *PUP14*, showing peak values in the *PUP14-GFP OX 4* line that also displays highest *PUP14* levels, and milder expression in the *PUP14-GFP OX 1* and *3* lines, which have moderate levels of total *PUP14*. *ARR6* and *ARR7*, primary targets of cytokinin signalling (Rashotte et al., 2003; To et al., 2004), are not affected in lines *1* and *2* (fold change ≤ 2). *ARR6* shows mild induction in line *3* only (2.2 x), whereas *ARR7* is slightly higher in lines *3* and *4* (2.46 x and 2 x, respectively) compared to wild-type (Fig. 2k). In summary, the expression profiling indicates that *PUP14-GFP* is expressed, which leads to higher total *PUP14* transcript levels. However, the stimulated expression is accompanied by an only marginal change in cytokinin transcriptional output in seedlings.

Structure-function analysis of PUP14 by site-directed mutagenesis

The lack of an unambiguous effect of *PUP14-OX* or *PUP14-GFP OX* on cytokinin signalling may be explained by posttranscriptional mechanisms regulation of PUP14 levels *in vivo*. To identify possible functional or regulatory residues, we subjected *PUP14* to targeted mutagenesis. We independently altered 3 well-conserved amino acid residues (Fig. 1, Appendix A2) putatively relevant for posttranslational regulation based on the amino acid properties. We thereby obtained inducible overexpressors of PUP14 P100G, PUP14 S333E, PUP14 S333C and PUP14 Q319A (*PUP14 P100G OX*, *PUP14 S333E OX*, *PUP14 S333C OX* and *PUP14 Q319A OX*, respectively). In embryos, induction of *PUP14 P100G OX* but rarely appeared to dampen cytokinin signalling (Fig. 3a), in contrast to *PUP14 Q319A OX* (Fig. 3b) and *PUP14 S333C OX* (Fig. 3c), which induced ectopic signalling. *PUP13 S333E OX* did not affect signalling (Fig. 3d). However, the effects were variable within lines and inconsistent across replications.

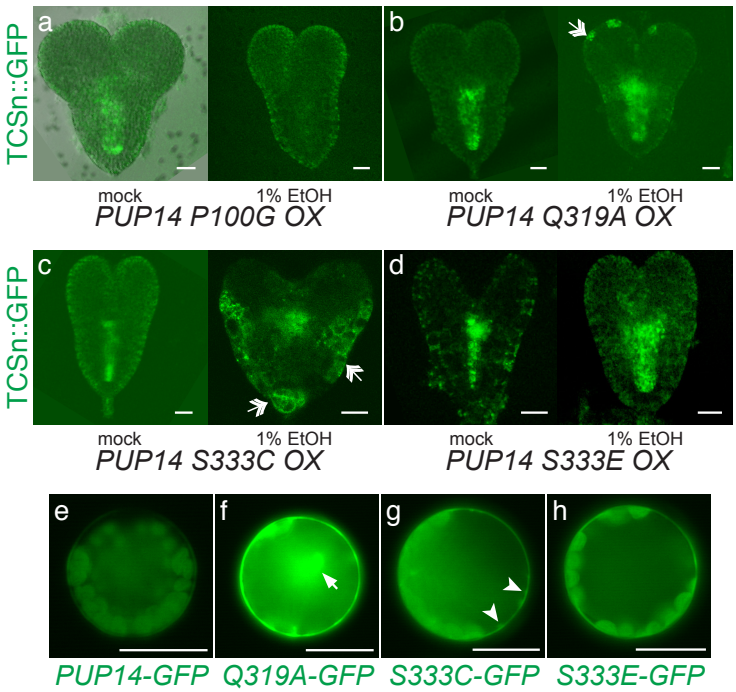

Fig. 3 | Analysis of mutant *PUP14* overexpression
(a-d) Embryos carrying mutant *PUP14* for inducible overexpression in *TCSn::GFP* lines after mock treatment (left panels) or ethanol induction (right panels).
(e-h) Protoplasts expressing GFP-tagged mutated *PUP14* variants under regulation of a strong constitutive promoter.
Double arrows indicate ectopic TCSn::GFP signal; arrows indicate signal from around nucleus; arrowheads indicate signal in cytoplasmic strands or ER.
Scale bars = 20 µm.

To determine subcellular localization of the mutant PUP14 versions, the respective GFP-protein fusions were prepared (*Q319A-GFP*, *S333E-GFP*, *S333C-GFP*) and analysed in transiently transfected protoplasts. The mutant PUP14-GFP variants were efficiently transfected and expressed as validated by the fluorescent signal that was readily found in ≥ 90 % of transfected protoplasts. For all variants, plasmalemma-localized signal was detected showing that plasma membrane targeting is still functional in the mutants (Fig. 3e-h). As expected, wild-type PUP14-GFP also localized to the plasma membrane, albeit with a markedly lower signal (Fig. 3e). Q319A-GFP and S333C-GFP also produced signals around the nucleus or in filamentous structures which is indicative of ER- or even cytoplasmic localization (arrow and arrowheads in Fig. 3e, f).
The observed patterns report that the introduced mutations do not affect localization at the plasma membrane. However, targeting to the plasma membrane appears less efficient for Q319A-GFP which also appeared in other locales.

DISCUSSION

amiR14_1 and amiR14_2 are efficient in targeting PUP14-HA

As we have shown already in Chapter 2, we have assessed plants with compromised *PUP14* function to determine the role of *PUP14* in cytokinin signalling. Because no suitable T-DNA insertion lines for *PUP14* were initially at hand, we have chosen to use artificial microRNAs (amiRs) for targeted *PUP14* gene silencing. The web-based tool WMD3 (http://wmd.weigelworld.org) allows the easy design of *amiRs* targeting single or multiple genes of interest and ranks *amiR* candidates based on parameters that are determined *in silico* (Schwab et al., 2006). In plants, negative regulation of gene expression by miRNA occurs primarily via target mRNA cleavage or translational inhibition (Brodersen et al., 2008), therefore the mere assessment of target gene transcript levels might not give full insight into the efficacy of the respective *amiR* construct. To include the inhibitory action of *amiR* on translation in the assessment of its potential, ETPamir detects changes in target protein levels. Furthermore, epitope-tagged proteins are used in this approach to circumvent the requirement of antibodies against the endogenous target protein, which might not always be at hand (Li et al., 2013, 2014). *PUP14* shares the highest sequence similarities with the lowly expressed *PUP15* (see Extended Data Fig. 1, Appendix A1) as the two are evolutionarily closely related (Gillissen et al., 2000; Jelesko, 2012). Redundancy within gene families is known to obscure potential effects of loss-of-function (Wagner, 1996), and we have therefore chosen to include *amiR* constructs targeting both *PUP14* and *PUP15* (*amiR14_1* and the non-characterized *amiR14_15*, Table 2, Appendix A2) besides the constructs that supposedly act on *PUP14* only (*amiR14_2* and *amiR14_3*). The evaluation of the reported ETPamir assays has shown that the *amiRs* affect protein levels of the target PUP14-HA within 18 hrs of induction. This result implies that the *amiRs* are effective in targeting *PUP14-HA* for downregulation either by mRNA cleavage or by translational inhibition. In our setup, *amiRs* and *PUP14-HA* are expressed from the same promoter and transcription of both should therefore be initiated with similar frequencies. Assuming that amiRNA processing and targeting occur at similar time scales as translation of mRNA into protein (Fluitt et al., 2007; Chakravarthy et al., 2010; Morozova et al., 2012), only little target protein will ever be made in the presence of highly efficient amiRNA. In our assay, we could still detect the targeted PUP14-HA, which indicates that the *amiR*-mediated downregulation is not equivalent to a gene knockout. As the *amiR* construct does not distinguish between endogenous and transgenic target mRNA, the apparent inefficiency in downregulation might however result from the higher number of total target mRNA (endogenous plus transgene copies) compared to amiRNA. To circumvent the incomplete silencing observed for concomitant expression of both *amiR* and target, inducible expression of target genes could be employed. Expression of the *amiR* construct from a constitutive promoter would give its production a head start, so that processed amiRNAs are already present once target gene expression is induced. This approach has also been suggested and implemented by the authors of the study describing ETPamir (Li et al., 2014).

In our assay, the PUP14-HA protein levels detected in the control sample are comparable across the experiment (Fig. 1a). Therefore we can presume that a steady state protein level is reached within the first 18 hrs and continuous production and turnover of protein ensure the maintenance of this level. The band intensities detected in the *amiR14_3*-expressing samples increase throughout the experiment and reach similar levels as the control within 38 hrs suggesting that *amiR14_3* is not sufficiently efficient to dampen expression of PUP14-HA beyond 24 hrs. Similarly, *PUP14-HA* levels appear to recover after 24 hrs in *amiR14_1* sample, which is not the case for *amiR14_2*, the most efficient *amiR* tested.
The ETPamir screening has allowed us to identify *amiR* efficacies in a semi *in vivo* system. However, the system is artificial and does not reflect the situation of stably transformed plants where amiRNAs target the endogenous transcripts (rather than endogenous plus transgene transcript copies). Moreover, in our case, *amiR* expression is elicited through an inducible system (Roslan et al., 2001) rather than being synchronised with target gene expression. Inherently, target protein may already be present upon *amiR* induction. Therefore, in the portrayed *in vivo* surroundings, the time scales in which *amiRs* are considered efficient depend also on protein turnover of the target; for proteins with a slow turnover rate, protein levels will remain close to steady state for longer. Accordingly, the induction length of *amiR* expression needs to be adjusted experimentally. In Chapter 3, we have found that PUP14-GFP levels are visibly decreased within 24 hrs of *amiR14_2* induction (Extended Data Fig. 2c,e, Appendix A1), validating that this represents a suitable time frame.
A different RNAi approach that has been reported to work efficiently in *Arabidopsis* and *Nicotiana benthamiana* is miRNA-induced gene silencing (MIGS) (Felippes et al., 2012). The technique relies on the endogenous miR173 and the production of trans-acting small interfering RNAs (tasiRNAs) (Felippes and Weigel, 2009). The processing of the MIGS precursor construct releases many tasiRNAs, which each recognize a different site in the target gene. Furthermore, because of the inherent feature of siRNAs that is known as transitivity (Himber et al., 2003; Voinnet, 2008), the MIGS approach has the potential of lowering target mRNA levels more efficiently than miRNAs. As an alternative to the *amiRNAs*, we have therefore tried to implement MIGS to target *PUP14.* However, with lines expressing a *PUP14 tasiRNA* construct, qRT-PCR analysis of seedlings revealed that *PUP14* levels were not affected. Since only few lines were assessed, I do not question the general efficiency and validity of the MIGS method.

PUP14 overexpression causes variable effects on cytokinin signalling output

Overexpression of *PUP14* has yielded a variation of effects on the TCSn expression pattern and therefore cytokinin signalling output (Fig. 2a-d). This might be the result of complex regulation and dynamic adaptation governing cytokinin signalling. On the other hand, the variable effects of overexpression might be explained by the non-uniform overexpression of PUP14 that we observed (Fig. 2e-j). However, the validation of *PUP14* overexpression depends on a different construct than the one used to determine its effects on cytokinin signalling (*PUP14-GFP OX* vs. *PUP14OX*, respectively). Hence, the patchy pattern observed with *PUP14-GFP OX* might not reflect the level of *PUP14OX* expression. The

two constructs could be transcribed and processed with different efficiencies. Equally, the resultant proteins might exhibit varying stabilities. A qRT-PCR study could compare transcription of *PUP14* in the two sets of lines, albeit transgenic and endogenous copies can currently only be distinguished in the *PUP14-GFP OX* line.
The seedling qRT-PCR assay (Fig. 2k) has revealed that in contrast to *PUP14* loss-of-function, *PUP14-GFP OX* does not affect expression of the early cytokinin target genes *ARR6* and *ARR7* (D'Agostino et al., 2000; Rashotte et al., 2003; To et al., 2004) except for line *PUP14-GFP OX 3* that displays a marginally induced expression of both *ARR6* and *ARR7*. The *PUP14OX* embryos have shown that *TCSn::GFP* signalling was affected in around 80 % of screened embryos (Fig. 2b-d). However, the change was not unidirectional; some embryos showed redistribution of the signal (Fig. 2b), while others displayed weakening or loss of reporter output (Fig. 2c,d). If *PUP14* overexpression affects seedlings accordingly, changes in the mere shape of signalling landscape are not detected by qRT-PCR measurements, as they do not necessarily translate into altered transcription levels of targets. Generally, observations made in the embryo cannot be directly applied to the seedlings; the embryos were individually assessed whereas seedlings were pooled for qRT-PCR which dilutes effects that are not highly penetrant. Moreover, the two sample types refer to very different developmental stages, obviously.

Structure-function analyses do not explicitly reveal posttranscriptional regulation

Preliminary *PUP14* overexpression experiments performed by Bruno Müller using a prior version of a *TCS* reporter (*TCSv2::tdTomato*) had indicated that the overexpression of PUP14 leads to a quenching of the cytokinin signalling output. This is in contrast to what I have observed for *TCSn::GFP* with *PUP14OX* (Fig. 2). The discrepancies between these experiments might be caused by differences in handling that inevitably occur when different people perform experiments. It is however possible, that different reporter properties have caused the diverging results as e.g. turnover rate of the reporter can influence how a dynamic or fluctuating signal is portrayed.
The fact that *PUP14OX* only caused mild effects on cytokinin signalling may be explained by potential posttranslational regulatory mechanisms that control PUP14 activity levels *in vivo*.
To address this question, we engaged in a risky side project that entailed the site directed mutagenesis of specific residues within PUP14 that are conserved across the *Arabidopsis thaliana* protein family (Fig. 1 , Appendix A2). A high degree of conservation is an indicator for a low tolerance towards mutations within these sequences. In other words, conserved sequences have withstood selection likely due to functional relevance of these residues. The peptide alignment of the PUP members reveals several conserved stretches from amongst which we chose 3 residues to target by mutagenesis. These included a proline (P100), a glutamine (Q319) and a serine (S333). Proline was selected due to its distinct side chain structure - a pyrrolidine - which has unique implications for protein structure (Li and Deber, 1994; Pavlov et al., 2009). Q had been misidentified by the author (EZ) who believed it was a glutamic acid (E) which would have been of interest due to its negative charge. As it turned out Q did not and still does not signify glutamate but rather glutamine.

Nonetheless, we included the Q319A mutant variant in the analysis. Incidentally, Qs have been found to represent uncommon glycosylation target sites at least in a human protein (Valliere-Douglass et al., 2010). Since glycosylation is known to affect protein stability, folding, and targeting (Knauer and Lehle, 1999), the Q319A was still of interest. Finally, with protein phosphorylation being the most abundantly identified posttranscriptional modification (Khoury et al., 2011), S333 was chosen as potential phosphorylation target site; it was altered into the phosphomimetic aspartic acid (E) and into a cysteine (C).
Analysis of the mutant variants indicates that these mutations do not interfere with plasma membrane localization. However, some variations were seen for Q319A-GFP which also showed signal around the nucleus that is likely the result of ER-localized protein (Fig. 3f). S333C-GFP was likewise identified in filamentous structure (Fig. 3g), which might be equivalent to ER or cytoplasmic localization. Because the ER constitutes the primary compartment in the secretory pathway (Hawes et al., 2015), the GFP signal obtained from these potential ER-sites might correlate with proteins, which are in the making. As neither the PUP14 wild-type nor the mutant sequences contain the canonical KDEL ER-retention signal (Stornaiuolo et al., 2003), unspecific localization as a result of saturated expression may be a valid explanation. On the other hand, signal from potential cytoplasmic strands that traverse the vacuole hint towards cleaving off of the GFP-tag.
The overexpression of the mutant PUP14 variants returned inconsistent and therefore inconclusive results, which did not allow us to infer functional properties for the targeted amino acids. Amino acid residues with high conservation across the *Arabidopsis* PUP family have been chosen for this analysis. If posttranslational mechanisms indeed operate on PUP14, it is possible that these regulations are unique within the family and therefore do not occur via the targeted conserved amino acids. Since it has been reported that most nonsynonymous mutations are deleterious to protein function (Sawyer et al., 2007), a more straightforward approach to identify whether regulatory mechanisms operate, would be the creation of transcriptional reporters. This may consist of the *PUP* promoter and a reporter protein such as the widely used GUS (Jefferson et al., 1987) or fluorescent proteins. We have cloned transcriptional reporter constructs for *PUP14* but could unfortunately not retrieve any transformed plants.

CONCLUSION

In this chapter, I have reported further experiments regarding *PUP14* function, which were not sufficiently conclusive to be added into the manuscript. I have addressed the efficacy of amiR constructs in downregulating PUP14-HA and studied the effect of *PUP14* overexpression on cytokinin signalling readout. Furthermore, I have made an effort to correlate specific well-conserved residues in PUP14 with unidentified regulatory aspects of PUP14 stability, targeting and function.

All in all, the obtained findings suggest that overexpression of *PUP14* is not a suitable approach to clarify its relevance in cytokinin signalling. This might not come surprising as hormone signalling is constituted of complex and multi-layered gene networks which can integrate a multitude of stimuli for rapid adaptation to changing conditions (Argueso et al., 2009; El-Showk et al., 2013; Pacifici et al., 2015; Sanz et al., 2015; Schaller et al., 2015). Post-translational modifications might counterbalance the overexpression by rendering supernumerary protein inactive to recover favourable signalling levels. Although the predicted PUP14 structure and the STRING database (www.string.org) (Szklarczyk et al., 2015) do not infer direct protein interaction, we would not exclude the possibility of dominant-negative effects caused by the altered stoichiometry in any hypothetic complex that includes PUP14 (Veitia, 2010). More in-depth analyses on the subject of regulation of PUP14 expression, stability and function is likely to shed light onto these matters.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. plants of the Columbia-0 (Col-0) ecotype were used as wild-type plants. For growth, seeds were surface sterilized for 18 min in 5% (v/v) bleach and 0.1 % (v/v) triton-X, washed 3 times in sterile ddH_2O and kept in the dark at 4 °C for a minimum of 2 days for stratification. For selection, seeds were sown on half-strength Murashige and Skoog (½ MS) medium with 2 % (w/v) sucrose, 0.8 % (w/v) phytagar and 2 mM MES pH 5.6 containing the appropriate antibiotic or herbicide for selection. Seeds on selection plates were placed into a Percival plant incubator (CU-36L6/D Percival Scientific Inc., Perry IA, USA) with 22°C and a 14/10 h light/dark regime with 120 mmol m^{-2} s^{-1}. For long-term growth, seedlings were transferred to soil and grown at 22°C and a 16/8 h photoperiod.

Agrobacterium *growth and stable transformation of plants*

Plant transformation was performed using *Agrobacterium tumefaciens* of the GV3101 (pMP90) strain. Plants were transformed as described (Clough and Bent, 1998). Transformations were carried out with plants of the Col-0 ecotype at bolting or up to an early flowering stage.

Plants carrying the *TCSn::GFP* reporter with inducible expression constructs (*PUP14OX*, *PUP14-GFP OX*, and mutant variants) were obtained via supertransformation of the *TCSn::GFP* plant line.

Ethanol induction of transgenes

To obtain deregulated expression of *PUP14* constructs from the ethanol inducible AlcR/AlcA promoter system (Roslan et al., 2001), embryos were treated as described previously (Müller and Sheen, 2008). Seedlings for qRT-PCR analysis were grown and induced as described in Chapter 3.1.

CLONING AND CONSTRUCTS

amiR *and* PUP *constructs for ETPamir*

The artificial microRNA (*amiR*) constructs were designed using the Web MicroRNA Designer WMD3 (www.weigelworld.org), and assembled by PCR amplification on pRS300 as template as described (Schwab et al., 2006) with the exception that newly designed oligo A and oligo B primers with LIC-adaptor sites were used. The primers for each construct are indicated in Table 1 in Appendix A2. The PCR amplicons were introduced into hbt95, a plant expression vector that contained the 35SC4PPDK promoter and the NOS terminator (Hwang and Sheen, 2001) by LIC (Aslanidis and de Jong, 1990). Cloning of *PUP14* into hbt95-HA was described in Chapter 3.1. *PUP4* was amplified from genomic Col-0 DNA using primers PUP4_LIC_F and PUP4_LIC_R and cloned into hbt95-GFP.

Overexpression constructs

For cloning of the inducible overexpression construct *PUP14OX*, *PUP14* was amplified from Col-0 genomic DNA with PUP14_LIC_F and PUP14_LIC_R primers, and cloned into DM7 (Zürcher et al., 2013) by LIC. For the *PUP14-GFP OX* construct, *PUP14-GFP* was amplified from *pPUP14::PUP14-GFP* in pCB302, which in turn was explained in Chapter 3.1. Sequences of primers used for cloning *PUP14-GFP OX* (PUP14_LIC_F and GFP_ LIC_R) are indicated in Table 1 in Appendix A2.

Site-directed mutagenesis of PUP14 *overexpression constructs*

Target amino acid residues for mutagenesis were chosen based on high conservation, SIFT BLink analysis (Ng and Henikoff, 2006), and inherent interesting side chain properties. Primer sequences used for targeted mutagenesis are given in Table 1 in Appendix A2. In a first step, the sequences from start to the mutation site, and from mutation site to stop were amplified with primers PUP14_LIC_F for start and PUP14_LIC_R for stop, paired with the relevant primer for mutagenesis. In a next step, the two amplicons were hybridized and extended to the full-length sequence by PCR using the PUP14_LIC_F and PUP14_ LIC_R primers. The full-length product was cloned into DM7 (Zürcher et al., 2013) by LIC. For the GFP-tagged mutant overexpressors, site-directed mutagenesis was carried out analogously using *pPUP14::PUP14-GFP* (Chapter 3.1) as template and GFP_LIC_R as reverse primer on the GFP 5'- end region. The full-length products were cloned into DM7 or hbt95 for stable and transient transformation, respectively.

Induction

To obtain deregulated expression from the ethanol inducible AlcR/AlcA promoter system (Roslan et al., 2001), embryos and seedlings were treated as described previously in Chapter 3.1.

ETPamiR

For transient expression, protoplasts of four-week-old wild-type *Arabidopsis* plants of the Col-0 ecotype were isolated as described (Yoo et al., 2007) with the following adaptations; vacuum infiltration was not performed in dark, protoplasts were counted after W5 wash and adjusted concentration was $5 * 10^5$ ml^{-1}, and W1 solution was supplemented with 15 mM sucrose. Transfections, and incubation were done as described (Li et al., 2014) with the indicated incubation times. SDS-PAGE analysis and Western blot were done as described (Li et al., 2014) with some modifications; protoplasts were resuspended in 40 µL 2x Laemmli buffer (126 mM Tris-HCl pH 6.8, 30 % (v/v) glycerol, 0.3 % (v/v) bromphenol blue, 4 % (w/v) SDS, 7.5 mM dithiothreitol (DTT)) and incubated at 95 °C for 8 min (Laemmli, 1970). Samples were aliquoted into 2 x 20 µL and used in discontinuous SDS-PAGE (Stacking gel: 4 % (w/v) polyacrylamide; resolving gel: 10 % (w/v) polyacrylamide) for protein separation. Primary antibodies, rabbit anti-HA (ab9910, Abcam, Cambridge, UK) and rabbit anti-GFP (632592, Clontech Laboratories, Saint-Germain-en-Laye, France) were added in 1:5'000 and 1:1'000 dilution, respectively and incubated o/N at 4 °C. Secondary antibody (goat anti-rabbit IgG, HRP-conjugated; AS09602, Agrisera, Vännäs, Sweden) was added in

1:5'000 dilution and incubated for 1 hr at RT. Tagged proteins were detected using the SuperSignal™ Dura West Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA USA 02451).

RNA isolation, cDNA synthesis and qRT-PCR analysis

To determine changes in transcript levels of cytokinin target genes and *PUP14*, induced *TCSn::GFP* and *PUP14-GFP OX* seedling were harvested into liquid nitrogen and ground using the MixMill (MM301, Retsch, Haan, Germany). An approximate 100 mg of frozen plant material were used for extraction with TRIzol® (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured using the NanoDrop ™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA 02451) and integrity was assessed by gel electrophoresis on a 2 % (w/v) agarose gel.

For cDNA synthesis, 2 µg of RNA were subjected to DNAse treatment with 0.5 µL DNAse I (recombinant, RNAse-free) in a total amount of 10 µL of 1 x incubation buffer (Ambion®/ Life Technologies Europe B.V., Zug, Switzerland). DNAse treatment was performed at 37 °C for 25 min. Prior to a 10 min heat deactivation at 75 °C, EDTA was added to a final concentration of 5 mM. For reverse transcription, Superscript III 1st Strand Synthesis Kit (Cat #18080-051, Invitrogen) was employed according to the manufacturer's instructions. Quantification of relative gene expression was done on an Applied Biosystems 7500 Fast Real- Time PCR System using SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies Europe B.V., Zug, Switzerland) or SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories AG, 1785 Cressier, Switzerland) according to manufacturers' recommendations. Final primer concentrations were 400 nM in a total volume of 20 µl. The relative values of the transcripts were normalized to *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A* (eIF4A, At3G13920) levels. Fold-changes for *PUP14*, *ARR6* and *ARR7* were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Relative expression levels for *eGFP* are stated as $2^{-\Delta Ct}$. *PUP14, ARR6* and *ARR7* primers are indicated in **Extended Data Table 1** in **Appendix A1**. *eGFP* primer sequences are 5'-ATCATGGCCGACAAGCAGAAGAAC-3' and 5'-GTACAGCTCGTCCATGCCGAGAGT-3'.

Microscopy

GFP expression in embryos was assessed by LSCM using either a Leica SP2 or Leica SP5 microscope. Protoplast images were captured using a Leica DM6000B epifluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland).

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4 Results *PUP* Family

Characterization of further *PUP* family members

INTRODUCTION

The Arabidopsis *PUP* family comprises 23 members of which presumably 21 encode proteins with predicted transporter structure. PUP1 had been previously implicated in transport of adenine derivatives including cytokinins (Gillissen et al., 2000; Bürkle et al., 2003; Cedzich et al., 2008). More recent research could show that PUP1, PUP2, PUP3 and 4 are able to mediate import of the vitamin B6 (pyridoxine) in a yeast heterologous expression system, and import into *Arabidopsis* mesophyll protoplast cells was demonstrated for PUP1 (Szydlowski et al., 2013). In Chapter 3.1, we have shown, that plasma membrane localized PUP14 imports tZ and that it functions in defining cytokinin signalling landscapes throughout development. In tobacco, the nicotine uptake permease NUP1 that belongs to the PUP family mediates transport of nicotine (Hildreth et al., 2011; Kato et al., 2014, 2015). In the monocot species *Oryza sativa*, there are 12 PUP-type transporters. A mutation in *OsPUP7* was found to increase cytokinin content in spikelets and OsPUP7- mediated transport of caffeine, another adenine derivative besides cytokinin, was determined in a yeast heterologous expression assay (Qi and Xiong, 2013). Apart from these studies, little has been published about members of the plant-specific *PUP* gene family. Within the scope of my PhD thesis, I have aimed at characterizing further *PUP* family members in terms of their expression pattern, subcellular localization, and function. An overview of the constructs cloned is given in Table 2 in Appendix A2.

RESULTS

Expression patterns

Based on the expression profiling of *PUP* family members in seedlings, embryos and protoplasts (Extended Data Fig. 1, Appendix A1), we chose to focus our further characterization on the moderately and, compared to *PUP14*, less ubiquitously expressed members *PUP1*, *PUP4*, *PUP5*, *PUP11* - *PUP13*, *PUP18*, *PUP21* and *PUP23*. Despite its very low expression levels in the contexts analysed, we also included *PUP15*, as it is the closest homologue of *PUP14* (Jelesko, 2012). We transformed plants with protein-GFP reporter constructs under the control of the native promoter for the above-mentioned genes to observe their expression patterns *in vivo*.

PUP1-GFP, PUP5-GFP, PUP12-GFP, PUP13-GFP and PUP21-GFP were not observed in considerable intensities in seedlings, leaves or embryos and were therefore not scrutinized further. PUP4-GFP and PUP11-GFP were both detected in the prospective cotyledons of the embryo (Fig. 1a, d), similarly to PUP14 expression and hence complementary to the cytokinin responsive domains (see Fig. 1b, Chapter 3.1). PUP4-GFP was furthermore detected in the SAM where it localizes to the cells of the peripheral zone (PZ) (Fig. 1b). During lateral root emergence, PUP4-GFP was found in anticlinally dividing cells of the pericycle (Fig. 1c). In contrast, PUP18-GFP was seen in epidermal cells of the leaves (Fig. 1e), and in the SAM, PUP18-GFP was observed limited to the L1 layer of the primordia (Fig. 1f). Furthermore, PUP18-GFP localized to the root cap and to a lesser extent also the root epidermis (Fig. 1g), while it was detected in epidermal cells overlying and surrounding the primordia during lateral root development (Fig. 1h), and again in the epidermal cell layer of the emerging (Fig. 1i) and growing lateral root (Fig. 1j). PUP23-GFP is also expressed in leaf epidermal cells (Fig. 1k), but only faintly in the root apex (Fig. 1l). PUP23-GFP is furthermore localized to epidermal cells during lateral root emergence (Fig. 1m) similar to PUP18-GFP.

Subcellular localization studies in heterologous systems

To determine subcellular localization, we have assayed the transient expression of GFP-tagged PUPs in onion and tobacco cells. Onion cells were cotransfected with *PUP1-*, *PUP4-*, *PUP11-*, *PUP15-* or *PUP23-GFP* and *PM-rk*, an mCherry-tagged plasma membrane marker (Nelson et al., 2007) and examined for expression using light microscopy. In all cases, the GFP signal colocalized with the mCherry-derived signal (Fig. 2a-f). The signals were however not limited to the plasma membrane but occurred also as web-like structures at the edges of the cells (PUP4-GFP, Fig. 2b), in cytoplasmic strands (PUP11-GFP, arrow in Fig. 2c), or throughout the cell (PUP15-GFP, Fig. 2e). To distinguish plasma membrane from vacuolar or ER localization, *ALMT9-GFP* and *pER-rk*, a vacuolar and ER marker, respectively (Kovermann et al., 2007; Nelson et al., 2007), were separately assessed besides *PM-rk* (Fig. 2g-i). All markers were recognized as fluorescent signal delimiting the margins of the transgene-expressing cells. In the case of ER localization portrayed by ER-rk, additional signal around the nucleus was recognized (Fig. 2g), whereas the vacuolar

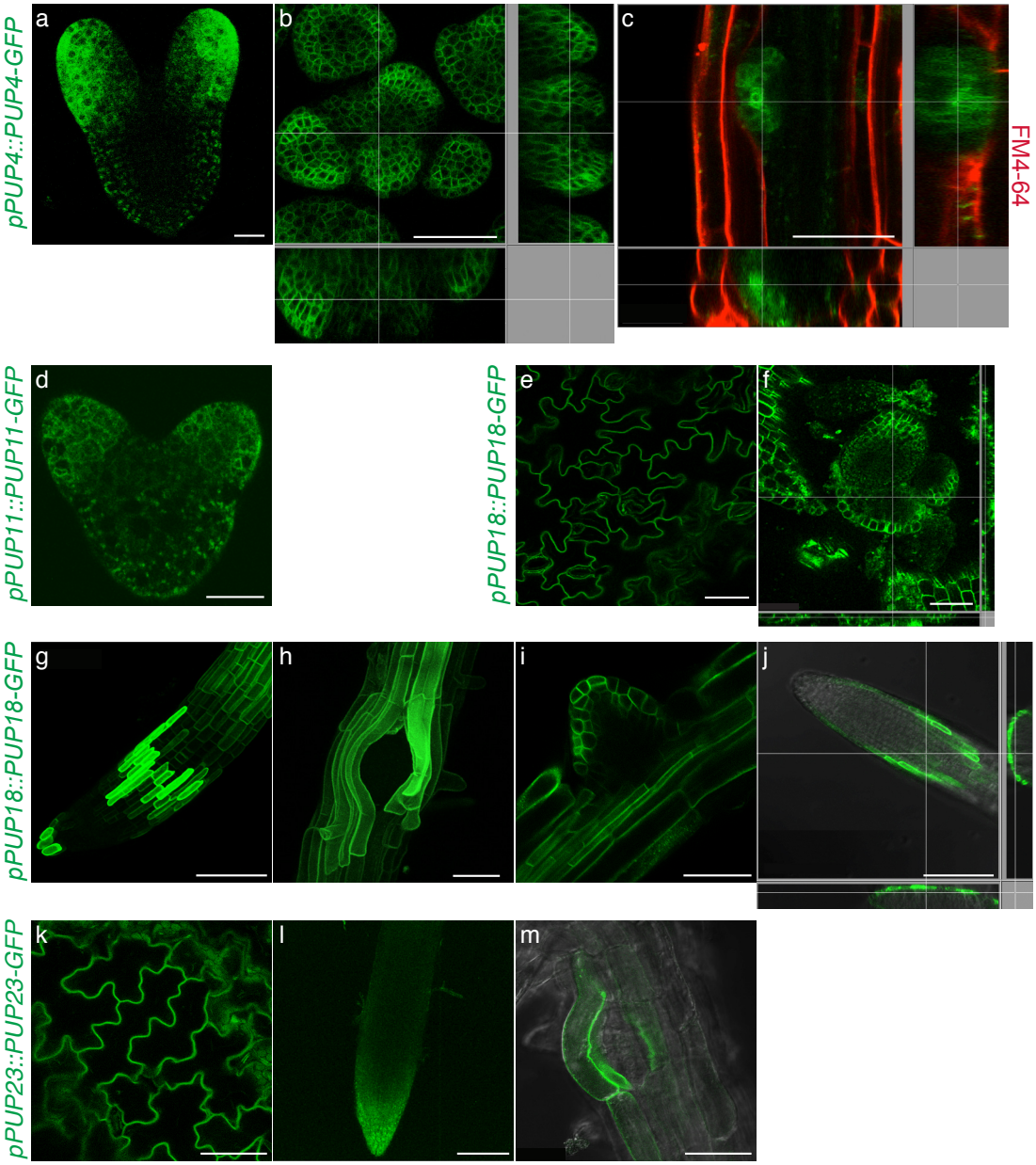

Fig. 1 | Expression patterns of selected *PUPs* in a subset of contexts
a-c) PUP4-GFP localization in the cotyledons of the embryo (a), the SAM (b), and in pericycle cells during lateral root emergence (c). PUP11-GFP localization in the cotyledons of the embryo (d). e-j) PUP18-GFP localization in epidermal cells of the leaf (e), the L1 layer of the SAM (f), the root cap (g), epidermal cells during lateral root emergence (h), root primordia and epidermal cells (i), and in the emerged lateral root (j). k-m) PUP23-GFP localization in leaf epidermal cells (k), in the RAM (l), and during lateral root emergence (m).
Pictures in g and h are maximum intensity projections of confocal z-stacks. Pictures in b, c, f, and j show x- and y- sections at indicated positions (white lines).
Scale bars in a, d= 20 µm, scale bars in b, c, e, f, h, i, k, m = 50 µm, scale bars in g, j, l = 100 µm.

marker could be distinguished by the demarcation of cytoplasmic strands (arrows, Fig. 2h), which is absent in the other two markers (Fig. 2g, i).

As an alternative localization study, tobacco leaf infiltration using inducible GFP-tagged overexpression constructs was performed (Fig. 2j-r). *PUP4-GFP OX*, *PUP10-GFP OX*, *PUP14-GFP OX*, *PUP15-GFP OX*, *PUP18-GFP OX*, *PUP21-GFP OX* and *PUP23-GFP OX* were transfected; *PM-rk* and *ER-rk* served as controls. For all of the constructs, fluorescent signals were readily detected indicating that the transfection and induction worked. However, the pattern of fluorescence produced by the overexpression was similar in all

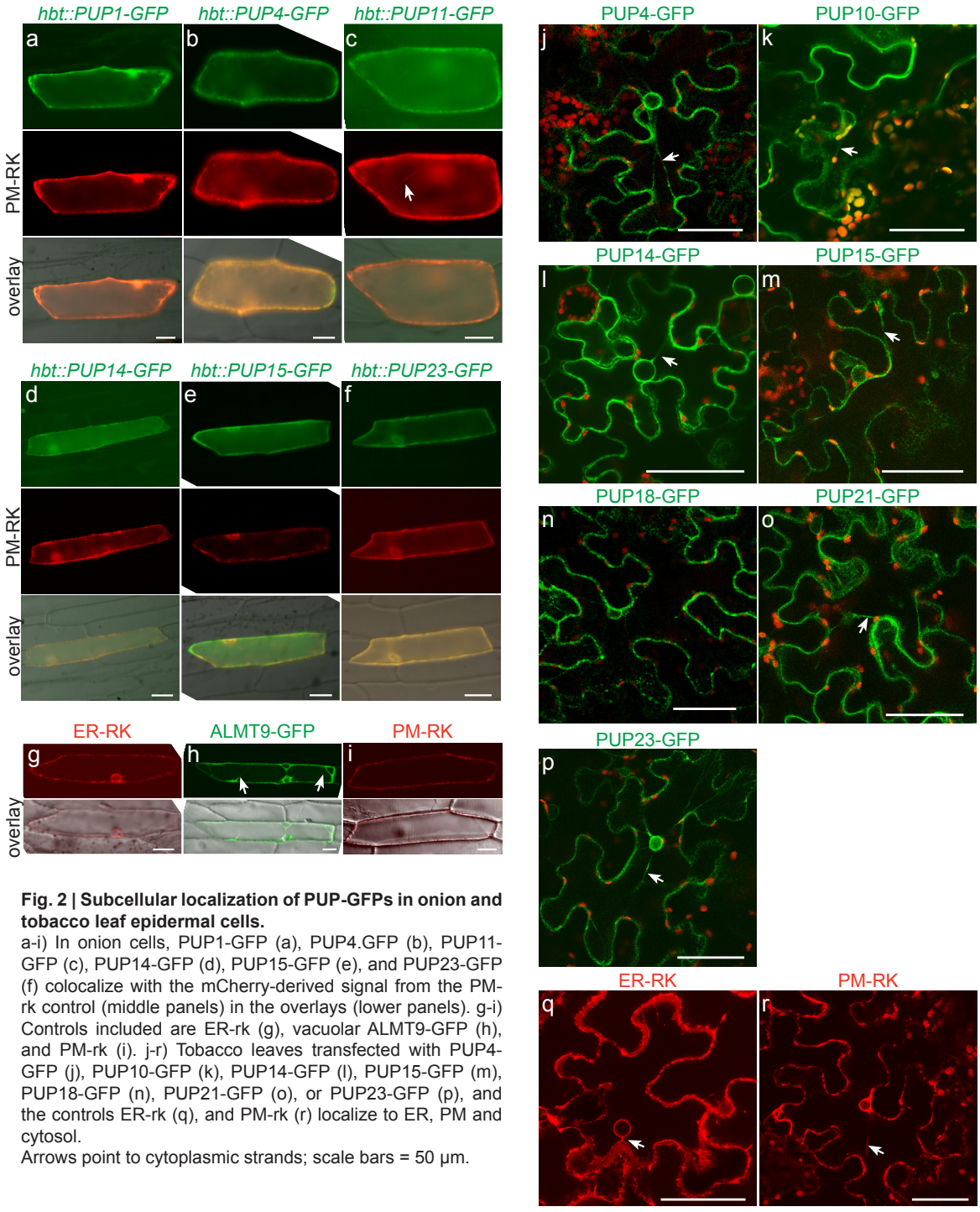

Fig. 2 | Subcellular localization of PUP-GFPs in onion and tobacco leaf epidermal cells.
a-i) In onion cells, PUP1-GFP (a), PUP4.GFP (b), PUP11-GFP (c), PUP14-GFP (d), PUP15-GFP (e), and PUP23-GFP (f) colocalize with the mCherry-derived signal from the PM-rk control (middle panels) in the overlays (lower panels). g-i) Controls included are ER-rk (g), vacuolar ALMT9-GFP (h), and PM-rk (i). j-r) Tobacco leaves transfected with PUP4-GFP (j), PUP10-GFP (k), PUP14-GFP (l), PUP15-GFP (m), PUP18-GFP (n), PUP21-GFP (o), or PUP23-GFP (p), and the controls ER-rk (q), and PM-rk (r) localize to ER, PM and cytosol.
Arrows point to cytoplasmic strands; scale bars = 50 µm.

cases, including the ER-rk and PM-rk controls (Fig. 2q,r), which were expected to cause differentially localized signal. Fluorescent signal was observed again as demarcation of the cells, as circles or webs surrounding the nucleus, and from cytoplasmic strands (arrows, Fig. 2j-r), which indicates plasma membrane, ER, and vacuolar or cytoplasmic localization, respectively. This pattern strongly hints towards unspecific targeting of the protein.
Overall, subcellular localization identification by the heterologous approaches was ambiguous and did not allow us to draw a definite conclusion about the PUP cellular residences.

Subcellular localization studies in protoplasts

In an alternative approach to determine the subcellular localization of the PUPs of interest, we used GFP-tagged PUP1, 4, 10, 11, 14, 15, 21 and 23 in a plant expression vector (hbt95) (Hwang and Sheen, 2001) for transient transfection in protoplasts. In protoplasts expressing *PUP1-GFP* or *PUP11-GFP*, no fluorescence signal was discernable. While

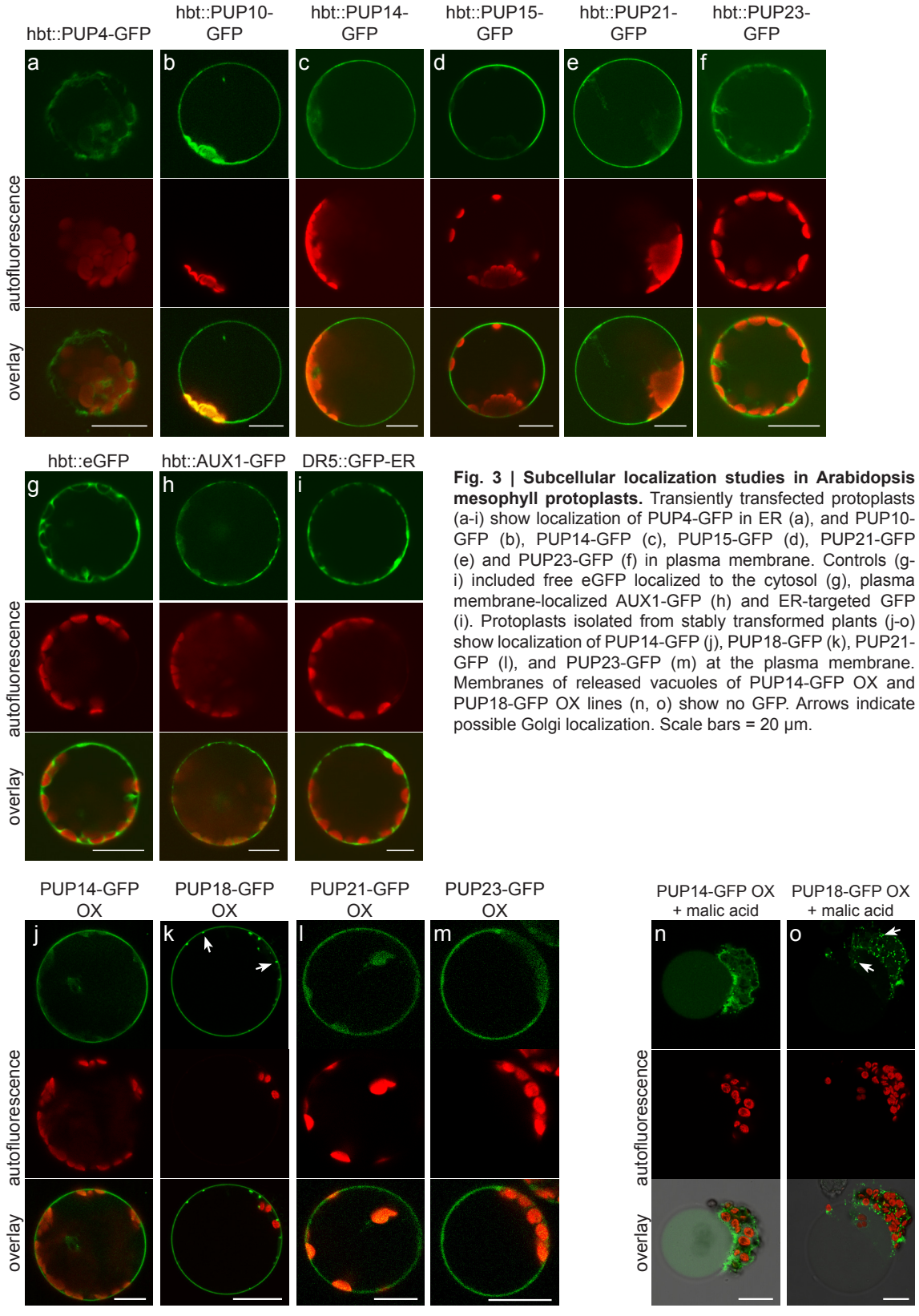

Fig. 3 | Subcellular localization studies in Arabidopsis mesophyll protoplasts. Transiently transfected protoplasts (a-i) show localization of PUP4-GFP in ER (a), and PUP10-GFP (b), PUP14-GFP (c), PUP15-GFP (d), PUP21-GFP (e) and PUP23-GFP (f) in plasma membrane. Controls (g-i) included free eGFP localized to the cytosol (g), plasma membrane-localized AUX1-GFP (h) and ER-targeted GFP (i). Protoplasts isolated from stably transformed plants (j-o) show localization of PUP14-GFP (j), PUP18-GFP (k), PUP21-GFP (l), and PUP23-GFP (m) at the plasma membrane. Membranes of released vacuoles of PUP14-GFP OX and PUP18-GFP OX lines (n, o) show no GFP. Arrows indicate possible Golgi localization. Scale bars = 20 µm.

PUP4-GFP signal could be seen as netlike structure suggesting ER-membrane localization (Fig. 3a), PUP10-GFP, PUP14-GFP, PUP15-GFP, and PUP21-GFP signal were observed as circular structures indicating plasma membrane localization (Fig. 3b-e). *PUP23-GFP* expressing cells however showed a pattern that was reminiscent of cytosolic localization (Fig. 3f), closely resembling free eGFP (Fig. 3g). AUX1-GFP (Swarup, 2004) and ER-targeted GFP were included to portray localization in the plasma membrane and ER-lumen, respectively (Fig. 3h, i).

In addition to transiently transfected protoplasts, protoplasts from stably transformed inducible *PUP14*, *PUP15*, *PUP18*, and *PUP21* and *PUP23* overexpression lines (*PUP-GFP OX*) were studied. Plasma membrane localized GFP-signal was observed in protoplasts derived from *PUP14-GFP OX*, *PUP18-GFP OX*, *PUP21-GFP OX* and *PUP23-GFP OX* lines (Fig. 3j-m). PUP18-GFP was furthermore visible as discrete, mobile dots, reminiscent of Golgi vesicles (Hawes et al., 2001) (Fig. 3k). In contrast, protoplasts from *PUP15-GFP OX* transgenic lines did not show a detectable GFP signal after induction with ethanol. As proteins localizing to the vacuolar membrane can give a similar visual impression as plasma membrane localized proteins, protoplasts were treated with malic acid to release the vacuole from the cell (Zhang et al., 2013). For *PUP14-GFP OX*- and *PUP18-GFP OX*-derived protoplasts, no signal could be seen in the membrane of released vacuoles. Instead GFP was detected in the cell debris surrounding the chloroplasts excluding the possibility of vacuolar localization. In malic acid-treated *PUP18-GFP OX* protoplasts, fluorescence was observed as individual spots interspersed in the chloroplasts (Fig. 3o), again indicative of Golgi targeting. The weak signal that was observed in intact protoplasts for PUP21-GFP (Fig. 3l) and PUP23-GFP (Fig. 3m) was no longer identifiable, neither in vacuoles nor in cell debris. In conclusion, the predominant subcellular localization for the assessed PUP members in protoplasts appears to be the plasma membrane. Additionally, PUP4-GFP was detected at ER-membranes, and PUP18-GFP in vesicles.

PUP FUNCTIONAL ANALYSES

Overexpression Studies

A previous study performed by Bruno Müller had indicated that the overexpression of *PUP10*, *PUP11* or *PUP18* induced wide ectopic expression of a formerly used cytokinin reporter, TCSv2::tdTomato, in heart-stage embryos (Fig. 4e). These experiments were repeated using the improved reporter *TCSn::GFP* (Zürcher et al., 2013) to qualitatively evaluate the effect of induced overexpression of *PUPs* on cytokinin signalling output. *PUP1*-, *PUP4*- *PUP10*-, *PUP11*-, *PUP15*-, *PUP18*-, *PUP21*- and *PUP23*- overexpressing plants (*PUPOX*) were analysed. *CKI1* overexpressing plants (*CKI1OX*) were treated in parallel as a control (see Fig. 1e, Chapter 3.1). No alteration of embryonic expression of TCSn::GFP was noted after induced *PUP1*, *PUP4*, *PUP11*, *PUP15* or *PUP23* overexpression. In contrast, *PUP10 OX*, *PUP18OX*, or *PUP21OX* caused deregulated *TCSn::GFP* induction in embryos (Fig. 4a-c). Compared to the gross ectopic signalling output reported by

TCSv2::tdTomato, the change in *TCSn::GFP* expression is moderate. This effect was also visible in the control *CKI1OX* where TCSv2::tdTomato signal had been obvious in virtually all cells, whereas TCSn::GFP signal was ectopic but patchy (Fig. 4d). In *PUP18OX* and *PUP21OX*, the ectopic signalling was limited to the L1 cell layer of the embryo while the endogenous signal remained unaffected (Fig. 4b, c) as opposed to embryos with the *PUP10 OX* construct, which showed a broadening of the endogenous signal (Fig. 4a). In summary, we found that overexpression of *PUPs* in embryos produces varying results depending on the *PUP* overexpressed. This might indicate different substrate specificities, functions or regulatory mechanisms acting on the differing *PUP* members. However, only few replicates were recorded for these experiments, hence the results need to be taken with reservations.

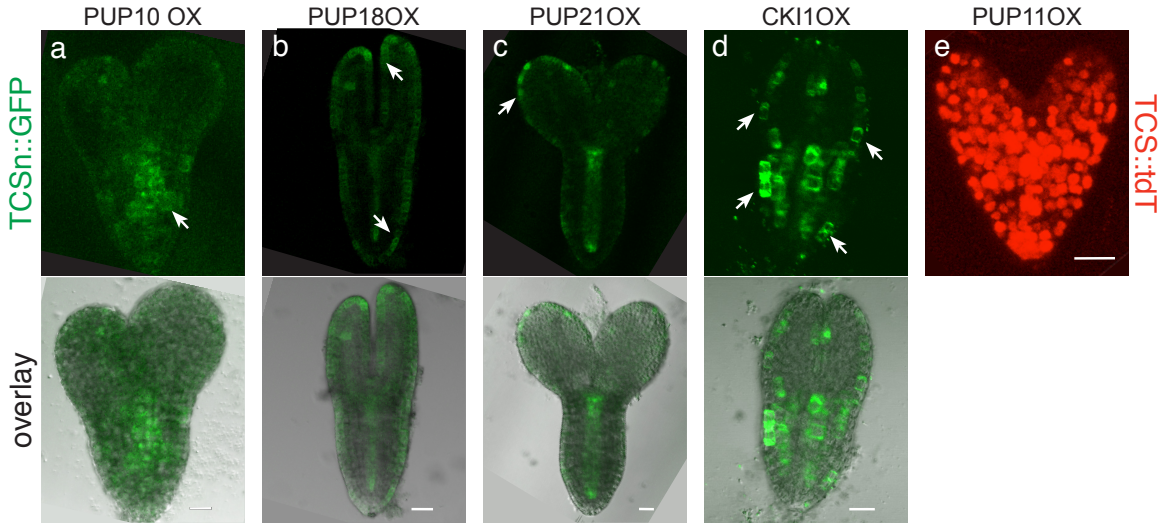

Fig. 4 | Effect of induced overexpression of *CKI1*, *PUP10*, *PUP18* and *PUP21* on *TCSn::GFP* reporter output in embryos. Overexpression of *PUP* constructs causes ectopic expression of *TCSn::GFP* (a- c). Ectopic expression is visible as broadening of the endogenous signal after *PUP10* induction (a), or as added signals in the L1 layer after induction of *PUP18* (b) or *PUP23* (c). *CKI1* overexpression yields patchy ectopic *TCSn::GFP* signalling in multiple loci throughout the embryo (d). Overexpression of *PUP11* in the previously used *TCSv2::tdTomato* line is given as a reference (e).
Lower panels are overlays of upper panels with recordings of transmitted light. Arrows indicate ectopic signalling in a-d; scale bars = 20 µm. Image in e) is a maximum intensity projection of confocal z-stacks recorded by Bruno Müller.

Validation of overexpression

In analogy to the procedure carried out for the validation of *PUP14* overexpression (Chapter 3.2), we sought to address the quality of inducible overexpression by checking PUP-GFP induction. Constructs for such inducible overexpression of GFP-tagged PUP members (*PUP-GFP OX*) were cloned for *PUP1*, *PUP4*, *PUP15*, *PUP18*, *PUP21* and *PUP23*, and transgenic plants are available for all but the *PUP1-GFP OX* construct. However, to date, no further efforts have been made to analyse these lines.

Δpup4 mutant analysis

A PUP4 T-DNA insertional mutant (*Δpup4*) was grown to assess the gene's function in development. Whereas the heterozygous *Δpup4* plants grew normally, the homozygotes were heavily delayed in growth, and remained weaker and smaller than the wild-type or

heterozygous plants. In accordance to their delayed development, these plants stayed green for a longer period. However, in a follow-up experiment, the mutant phenotype could not be reproduced.

Δpup11 mutant analysis

Previous work by Anja Schmidt in the lab of Ueli Grossniklaus had shown that homozygous *Δpup11* mutant embryos abort early in development (Anja Schmidt and Ueli Grossniklaus, unpublished). To corroborate the importance of *PUP11* during early development, we chose to complement *Δpup11* plants by transformation with *pPUP11::PUP11-GFP*. In the T1 and T2 generation, recovered plants were either wild-type or heterozygous in the *Δpup11* locus. Likewise, crosses of *Δpup11* and *pPUP11::PUP11-GFP* plants did not yield *Δpup11* homozygous offspring. Since the GFP-tag might interfere with proper PUP11 targeting or function, we sought to complement *Δpup11* lines with a *PUP11* construct spanning the genomic locus from promoter to the 3' UTR. As for the GFP-tagged lines, no plants homozygous for the *Δpup11* T-DNA insertion could be retrieved from these transformations indicating that neither *pPUP11::PUP11-GFP* nor the genomic *PUP11* construct can complement the *Δpup11* mutation.

amiR mutant analyses

As redundancy among protein families are common and because T-DNA insertion lines were not available for all members of interest, we chose to employ an RNA interference (RNAi) aproach that allows the simultaneous downregulation of several PUPs. We have designed inducible *amiRs* to target *PUP3*, *PUP10*, *PUP12*, *PUP13* and *PUP21* (*amiR_B*), *PUP17* and *PUP18* (*amiR_C*), and *PUP19* and *PUP20* (*amiR_D*, not shown). *AmiR* constructs were transformed into *TCSn::GFP* plants to assess the effect of *PUP* downregulation on cytokinin signalling output (**Fig. 5**). In heart-stage embryos, ethanol-induced *amiR_B* expression causes ectopic signalling in the L1 layer (**Fig. 5b**), which is not detected in

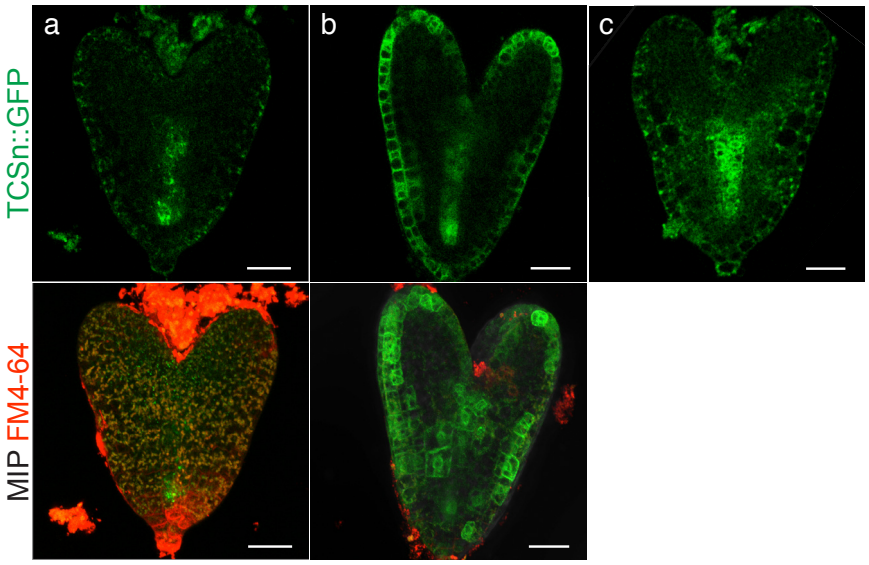

Fig. 5 | Effect of amiR-mediated PUP downregulation on TCSn::GFP expression in the embryo. Heart-stage embryos of *amiR_B* (a, b) after mock-treatment (a), and ethanol-induction (b). Heart-stage embryo of ethanol-induced *amiR_C* (c). Lower panels are maximum intensity projections (MIP) of z-stack sections of embryos in upper panels. Scale bars = 20 µm.

the mock-treated control embryos (Fig. 5a). In contrast, the downregulation of *PUP17* and *PUP18* by induced *amiR_C* expression does not have an effect (Fig. 5c). These preliminary results suggest that besides *PUP14*, also the sum of PUP3, PUP10, PUP12, PUP13 and PUP21 action is required to limit cytokinin signalling in the heart-stage embryo. In contrast, *PUP18* and *PUP17*, are not required to determine the embryonic cytokinin landscape. This is in accordance with their low expression in embryonic tissue (see Extended Data Fig. 1, Appendix A1).

Protoplast transport assay

PUP1 has been previously shown to mediate transport of adenine and derivatives (Gillissen et al., 2000; Bürkle et al., 2003; Cedzich et al., 2008). In addition, we showed in Chapter 3.1 that PUP14 can similarly import tZ and that uptake can be inhibited by excess of isopentenyl adenine (iP), benzyladenine (BA), or adenine. To determine transport capabilities of PUP family members of interest, a protoplast uptake experiment was performed. Isolated protoplasts were transiently transfected with *PUP1*, *PUP4*, *PUP10*, *PUP11*, *PUP15*, *PUP18* or an empty vector (mER) and incubated with ^{14}C-labelled tZ for 30s, 5, 10 or 15 minutes. Levels of imported labelled trans-zeatin were measured by scintillation counting. In all cases, an increase in tZ levels over time was evident. Expression of *PUP1*, *PUP4*, *PUP10* and *PUP15* led to accelerated uptake as indicated by the higher levels of label at the later incubation times. Although import in *PUP11*- or *PUP18*- transfected protoplast was still enhanced compared to the control, the uptake-stimulating effect for these two constructs was less pronounced (Fig. 6).

This preliminary experiment suggests that various PUPs can facilitate uptake of tZ into protoplasts.

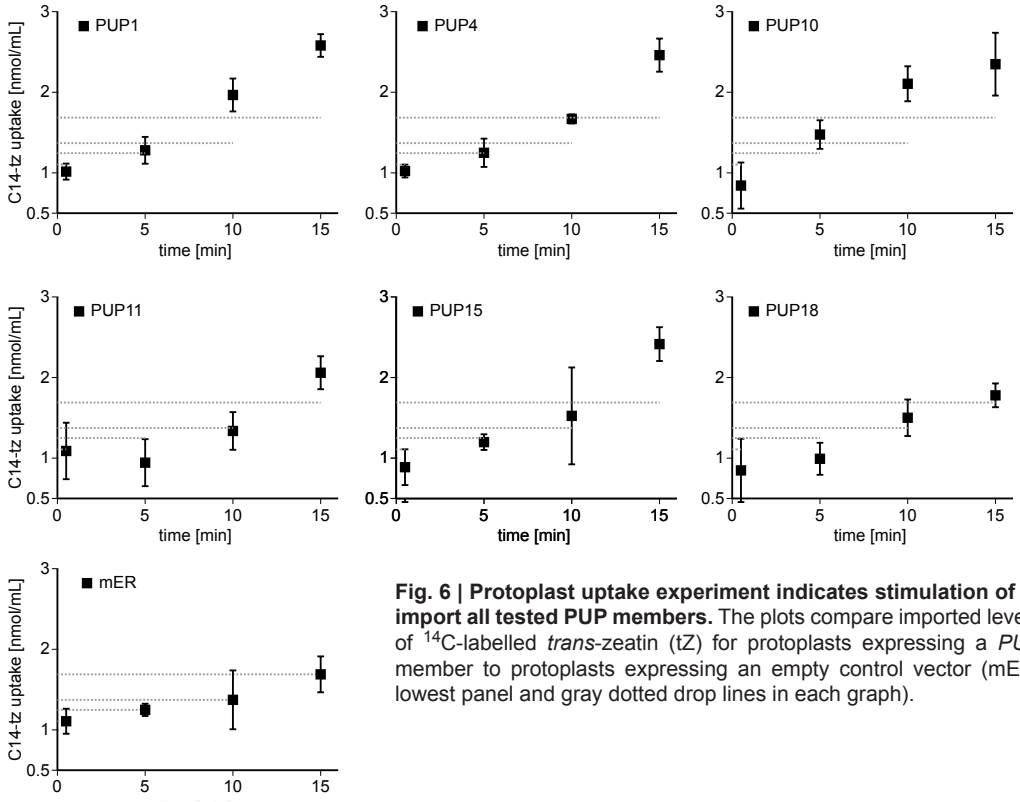

Fig. 6 | Protoplast uptake experiment indicates stimulation of tZ import all tested PUP members. The plots compare imported levels of ^{14}C-labelled *trans*-zeatin (tZ) for protoplasts expressing a *PUP* member to protoplasts expressing an empty control vector (mER, lowest panel and gray dotted drop lines in each graph).

DISCUSSION

Expression patterns suggest diverging roles for PUPs

To determine the expression patterns of individual *PUP* members, we used GFP-protein fusions of PUP members to visualize expression. Because of our strong interest in embryo development, we have first checked expression therein. In addition, expression in other, more easily accessible structures such as leaves or the seedling root tip was assessed. It is however probable that dynamic or narrow expression in specific tissues escaped our notion. Accordingly, the failure to detect PUP1-GFP, PUP5-GFP, PUP12-GFP and PUP13-GFP may arise from not assessing the right tissue locale at the right time. The results obtained from the analysis of the GFP reporters are consistent with my qRT-PCR data and published microarray studies (see **Extended Data Fig. 1**, **Appendix A1**, Belmonte et al., 2013; Yadav et al., 2014; Adrian et al., 2015). Obviously, also these expression data might not seize signals from very dynamically regulated genes. Our prioritized aim was however, to determine generic patterns of expression rather than identifying circumstantial responses. Hence, the two sets of expression data - portraying protein and transcript levels - mutually affirm each other.

For *pPUP4::PUP4-GFP*, *pPUP11::PUP11-GFP*, *pPUP18::PUP18-GFP* and *pPUP23::PUP23-GFP*, we could establish tentative expression patterns. The embryonic patterns of *PUP4* and *PUP11* expression are reminiscent of the ones discovered for *PUP14* as they also exhibit complementarity with cytokinin signalling. For PUP4-GFP, this also holds true for expression in the SAM (**Fig. 1b**), where expression was prominent in the peripheral zone (PZ), but not in the organising center (OC) where *TCSn::GFP* is expressed (see **Fig. 4b, c**, **Chapter 2**, and **Fig. 2b**, **Chapter 3.1**), neither in the central zone (CZ) overlying the OC (Kerstetter and Hake, 1997). Remarkably, LOG4 and therefore cytokinin activation are restricted to the L1 layer with highest expression in L1 of the CZ (Chickarmane et al., 2012). Together, this indicates that PUP4 is negatively correlated with cytokinin production and activity. This pattern is repeated during lateral root emergence (**Fig. 1c**), where PUP4-GFP locates to the inner and outer layer of the lateral root primordium (Malamy and Benfey, 1997). In comparison, TCSn::GFP signal is absent from these cells (see **Fig. 4f**, **Chapter 2**, and **Fig. 1m**, **Chapter 3**). With PUP11-GFP also localizing to the embryonic cotyledons, it is likely that the function we uncovered for *PUP14*, namely definition of cytokinin signalling domains, can be applied to other *PUP* members.

Expression of PUP18-GFP does not overlap with *TCSn* expression either, but no longer fulfils the striking pattern of complementarity; it is found in the epidermal cell layer of leaves, SAM, lateral roots, roots, and subsets of the columella and lateral root cap cells (**Fig. 1e-j**). As outermost layer, the epidermis represents the boundary between the plant and its environment. This apparent correlation of PUP18-GFP localization and direct contact with the outside space is also reflected in protoplasts. In these isolated cells, *PUP18* showed its highest expression (see **Extended Data Fig. 1**, **Appendix A2**). It is however unclear, whether this is a result of induction of *PUP18* to match the new circumstances of environmental exposure. Alternatively, *PUP18* might be expressed also in mesophyll cells prior to

protoplast isolation. Either way, an obvious role for PUP18 might lie in taking up nutrients from the surroundings. However, if PUP18 activity were limited to taking up compounds from the environment, one might expect polarized localization (Barberon and Geldner, 2014), which we did not notice. Interestingly, root epidermal cells that are directly pushed apart by the emerging lateral root (Vermeer et al., 2014), typically showed an increased expression of PUP18-GFP (**Fig. 1h**), indicating a relevance for *PUP18* function in these cells. In the epidermis and during lateral root initiation, PUP23-GFP showed overlapping expression with PUP18-GFP. In the root apical meristem however, PUP23-GFP is only weakly expressed and less distinctly localized to the columella root cap suggesting that PUP18 and PUP23 have undergone subfunctionalization (Force et al., 1999).

Heterologous expression systems for subcellular localization analysis did not allow unambiguous determination of PUP locales

PUPs are predicted to carry 10 membrane-spanning domains (Gillissen et al., 2000), which inevitably targets them to membranes. To determine whether they are localized to the plasmalemma or to endomembranes, we assessed PUP-GFPs in *Arabidopsis* protoplasts, and in onion and tobacco epidermal cells. In these analyses, the fusion proteins were under the control of strong, ubiquitous promoters. This is particularly helpful when driving expression in the heterologous systems, as we do not know whether the endogenous promoter can efficiently recruit the corresponding homologous transcription factors. Also in protoplasts, which are derived from only a fraction of the different cell populations in a plant, it is not certain that an endogenous gene will be expressed. However, due to the nature of the secretory pathway, strong expression of membrane or secreted proteins has the drawback that subcellular localization might not be deduced unambiguously (Denecke et al., 2012). Especially in tobacco cells, I have made the frequent observation of ER-derived signal also in the PM-rk control, which should portray plasma membrane localization (Nelson et al., 2007). This demonstrates that the levels of protein production are so high that they can be detected “in the making” at the ER-membrane. Also, ER- retention of improperly processed or misfolded protein through quality control mechanisms can cause strong ER-derived signal (Moore and Murphy, 2009). Shorter incubation times, or the assessment of less strongly expressing cells might have alleviated this issue (Denecke et al., 2012).

Onions constitute a very amenable tool for localization studies and have been used in a large number of studies (Collings, 2013). In onion cells, the lack of chloroplasts can however be regarded as a double-edged sword. On one hand, it rids the most prominent source of autofluorescence coming from chlorophyll. This is especially nice when using red fluorophores such as mCherry. On the other hand, lack of chloroplasts is accompanied by supremacy of the vacuole; cytosol and all other organelles, including the nucleus and the ER are pushed aside making it difficult to distinguish between localization in the cytosol, or vacuolar, ER and plasma membrane (**Fig. 2g-l**). In protoplasts, the ensemble of chloroplasts defines vacuole-free space allowing a more ascertained distinction between the vacuole, cytosol and ER, the latter of which often forms net-like structures around nucleus and chloroplasts (**Fig. 3g-i**). Obviously, in terms of protein processing, the environment provided

by *Arabidopsis* protoplasts represents the *in vivo* situation most closely, whereas targeting in heterologous systems might be faulty due to non-conservation of signal sequences (Collings, 2013).

PUPs localize to the plasma membrane with some deflections

Despite the unexpected difficulties encountered in the localization studies, we could determine the plasma membrane as target site for PUPs with a high degree of certainty. Not only the use of protoplasts as expression system (Fig. 3b-e, j-m) was critical in this finding, but also the analysis of fusion proteins *in vivo* (Fig. 1). Plasma membrane localization is in agreement with localization of the tobacco PUP1 homolog NUP1 (Hildreth et al., 2011; Kato et al., 2015). Nonetheless, we have observed some variations. Namely PUP4-GFP and PUP18-GFP additionally occurred at ER-membranes and Golgi vesicles in protoplasts, respectively. Whereas we detected PUP4-GFP *in vivo* primarily at the plasma membrane, PUP18-GFP in plants could also be seen in vesicles (e.g. Fig. 1i, epidermal cells next to lateral root), suggesting that PUP4-GFP at ER-membranes in protoplasts is an artefact of overexpression. In contrast, PUP18 might indeed be engaged in endocytic recycling as is known e.g. for PIN1 (Marhavý et al., 2011). It has been reported though that strong expression of plasma membrane proteins can lead to deposition of this proteins in newly formed vesicular structures (Brandizzi et al., 2002; Saint-Jore-Dupas et al., 2004). It is noteworthy, that the GFP-tagged PUP18 homolog, PUP23 (expressed from pPUP23::PUP23-GFP or 35S::PUP23-GFP) was less strongly expressed and did not display localization in vesicles (Fig. 1k-m, 3m) indicating that vesicular deposition of protein is a result of strong expression. As for PUP23-GFP driven from a strong, constitutive promoter (*hbt::PUP23-GFP*), the signal detected suggested cytosolic localization (Fig. 3f). This is in turn indicating that GFP is cleaved off from PUP23 as the hydrophobic domains of PUPs inevitably targets them to membranes (Gillissen et al., 2000). Similarly, fluorescent signal in the vacuoles of onion cells expressing PUP15-GFP (Fig. 2e), and protoplasts expressing 35S::PUP14-GFP (Fig. 3n) can be explained by processing of the full-length tagged protein into its two constituents.

Overexpression of PUP18 and PUP21 have weak effects on cytokinin signalling output

In analogy to the procedure carried out for PUP14 characterization in Chapter 3.1, I have checked the effects of *PUP* overexpression on *TCSn::GFP* signalling output in embryos (Fig. 4), verified *PUP* overexpression and did site-directed mutagenesis for individual PUPs (not presented). Contrary to what was observed with the previous version of the TCS reporter, TCSv2::tdTomato (Fig. 4e), *PUP* overexpression did not consistently affect cytokinin signalling pattern (Fig. 4a-d). Rather, overexpression of most of the tested *PUPs* (*PUP1*, *PUP4*, *PUP11*, *PUP15* or *PUP23*) did not change *TCSn::GFP* signalling under the conditions used.

PUP10 overexpression however resulted in a weak broadening of the signalling domain (Fig. 4a), whereas *PUP18OX* or *PUP21OX* caused ectopic signalling in the L1 layer of the embryo (Fig. 4b,c). Remarkably, the signal in the provasculature was not affected.

As I have reported above, we found PUP18-GFP localized to the epidermal layer in many

if not all instances (Fig. 1e-j). The appearance of ectopic TCSn::GFP precisely in the outer layer of the embryo is striking. It can only be speculated whether the recurring theme of L1-specific expression respectively induction is correlated or merely coincidental. As for *PUP14*, the overexpression did not allow us to draw a terminal conclusion.

Knockdown mutant reveals embryonic function for moderately expressed PUPs

To downregulate expression of *PUP3*, *PUP10*, *PUP12*, *PUP13* and *PUP21* concomitantly, I have employed an inducible *amiR* construct (*amiR_B*). In *amiR_B*-expressing embryos, we could observe that TCSn::GFP was ectopically switched on predominantly in epidermal cells but also in cells of inner layers (Fig. 5b). Again, as observed for *PUP18OX*, signalling output in the provasculature was not grossly altered in most samples examined. In the group of *PUPs* targeted by *amiR_B*, each individually only displays low to intermediate transcript levels (see Extended Data Fig. 1, Appendix A1), which explains why a qRT-PCR assay to determine the degree of downregulation was not successful (not shown). Nonetheless, their mutual loss has an effect on signalling suggesting that one, a few, or the collective of the targeted *PUPs* have a role in limiting cytokinin signalling similar to *PUP14* function. Unfortunately, their low expression rates made it difficult for us to detect their expression pattern, hence I can not make any assumption to whether they are complementary to cytokinin output as is the case for *PUP14*.

The second investigated amiR, *amiR_C*, targets *PUP17* and *PUP18*, neither of which is considerably expressed in the embryo (see Extended Data Fig. 1, Appendix A1). Therefore it is not surprising that their loss in embryos does not have an impact on cytokinin signalling (Fig. 5c). For PUP18, the protein expression data reveals that it is not mutually exclusive with TCSn::GFP and therefore cytokinin signalling. This hints towards a role for *PUP18* that differs from defining cytokinin signalling landscape. Accordingly, it might seem bold to assume that TCSn::GFP would even be altered in the *amiR_C* mutant. Nonetheless, as we have seen for the *PUP18OX*, deregulation of *PUP18* can have an effect on signalling (Fig. 4b). But, how this effect comes about, will be the subject of further investigation.

T-DNA mutant analysis

We have ordered T-DNA insertion lines from the public databases to check whether the loss of a particular *PUP* impairs correct growth and development. Anja Schmidt who has investigated the *Δpup11* T-DNA lines determined early embryonic abortion for the homozygous mutant (Anja Schmidt and Ueli Grossniklaus, unpublished). Naturally, we were keen on validating that the phenotype was due to the loss of *PUP11* function. Unfortunately, our trials to complement these lines by *pPUP11::PUP11-GFP* and the genomic *PUP11* locus were not successful. The underlying reason might be that the *PUP11* transgenes or gene products are not correctly transcribed or processed. The average *Arabidopsis* promoter was reported to measure 500 bp (Korkuc et al., 2014). It is therefore unlikely that we missed *PUP11* upstream regulatory elements required for expression as we have included more than 1500 bp as promoter. The plants had been selected on the basis of their resistance towards BASTA which indicates that silencing due to integration into heterochromatic region (Gelvin and Kim, 2007) did not underlie the lack of complementation.

It has been reported that each Salk line contains ~1.5 insertions (Alonso, 2003) and according to the FAQ section on the Gabi-Kat homepage "[…] there are many lines with two or even more insertions" (http://www.gabi-kat.de/faq.html#faq35). It is therefore possible that the phenotype observed is not caused by the insertion in the *PUP11* gene. Embryonic abortion has been however identified in two independent insertion lines, which is usually considered sufficient to attribute a phenotype to the function of the disrupted gene. Current efforts undertaken by my colleague James Jingchun Liu are aiming at resolving the conflicting data.

The assessed *Δpup4* T-DNA insertion mutant had produced an interesting phenotype in which growth of the homozygous plant was retarded. Such a general growth defect is recapitulated in several cytokinin signalling mutants including higher-order mutants of *AHKs* (Nishimura et al., 2004; Riefler et al., 2006), *AHPs* (Deng et al., 2010), or type-B *ARRs* (Argyros et al., 2008). Unfortunately, the phenotype was not observed in a repetition of the experiment under similar conditions, indicating that the observed defects were coincidental or that the plants had compensated for *PUP4* loss through adaptation.

PUPs are transporters of trans*-zeatin*

We have determined that protoplasts transfected with *PUP1*, *PUP4*, *PUP10*, and *PUP15* stimulate import of labelled tZ in mesophyll protoplasts (Fig. 6). The initial study that reported the transport function for PUP1 had identified *PUP1* through a yeast complementation study that aimed at complementing an adenine uptake deficient yeast strain with an *Arabidopsis* cDNA library (Minet et al., 1992; Gillissen et al., 2000). Interestingly, only *PUP1 – 4* had been recovered although we see that also the other tested PUPs have the capacity to act as transporters. Obviously, the transport substrate used in the screen was not tZ but adenine. Together, this data demonstrates the broader substrate specificity of PUP1 - 4 compared to PUP10 and PUP15. Amongst others, PUP1-mediated uptake was shown for kinetin, caffeine (Gillissen et al., 2000), tZ, iP (Bürkle et al., 2003), and pyridoxine (Szydlowski et al., 2013). It is however noteworthy that in the yeast complementation study to rescue pyridoxine uptake deficiency, the use of the same cDNA library (Minet et al., 1992), had not retrieved *PUP1*. Only the direct cloning and transformation with *PUP1* cDNA successfully established PUP1 as pyridoxine transporter (Szydlowski et al., 2013). This shows that not all candidates are detected by the cDNA complementation approach. Considering the low abundance of many *PUP* transcripts, one might not be surprised that many PUPs were not recovered in the mentioned yeast complementation screens.

PUP11 and PUP18, which showed a less strong stimulation in our protoplast uptake experiment, presumably have low affinities towards tZ under the applied conditions. Since we have noticed that PUP18 and cytokinin signalling do not show any correlation in regards to their location, this finding confirms our suspicion that PUP18 preferentially transports other substrates than tZ. To ascertain the substrate specificities it will be indispensible to check uptake rates for further compounds and to run competition experiments. Furthermore, addition of ATP, protonophores or the variation of pH as done previously for PUP1 (Bürkle et al., 2003), should give insights into the energy dependency of the individual family members' functions.

CONCLUSIONS

In this chapter, I have focused on *PUP* members apart from the previously discussed *PUP14*. Based on expression levels, I have chosen several of the 23 family members for characterization. With GFP-tagged protein fusion constructs, I have checked the *in vivo* distribution of proteins and determined the subcellular localization in endogenous and heterologous settings. In order to identify roles of the various *PUP* members, I have tried overexpression and mutant analyses. Moreover, transport potential of the selected members were assayed in a protoplast uptake experiment.

These efforts to identify functions for the individual *PUP* genes went into several directions, but many aspects were only tackled superficially due to the tremendous workload imposed by the large number of interesting candidates. Continued research focusing on the *PUP* family will likely uncover more roles for these genes.

MATERIALS AND METHODS

Plant lines and growth conditions

T-DNA insertion lines in PUP11 (*Δpup11*) are GK-421B11.01 (N348765) (Kleinboelting et al., 2012) and SALK_112079C (N661092) (Alonso, 2003) and were kindly provided by Anja Schmidt. T-DNA insertion line in PUP4 is GK-348G04.09 (N321554) and was ordered from NASC. Genotyping primers are indicated in Table 1 in Appendix A2.

Constructs

Full tables of used primers and constructs are given in Appendix A2 in Table 1 and 2, respectively.

For GFP translational reporter lines, *PUPs* were amplified from Col-0 genomic DNA and cloned into pCB302 by LIC (Aslanidis and de Jong, 1990). *PUPs* in the plant expression vector hbt95 for protoplast localization and mircoprojectile bombardment of onions were amplified from Col-0 genomic DNA with PUPX-LIC primers (X = respective PUP) and inserted into the hbt95 by LIC (Hwang and Sheen, 2001). The same procedure using the same primers was applied for cloning into the binary vector DM7 for overexpression in stably transformed *Arabidopsis* and for tobacco leaf infiltration. GFP-overexpressor lines were amplified on the respective pPUP::PUP-GFP reporter lines in pCB302. Sequences were cloned to include the coding sequence of the respective *PUP* and the entire GFP coding region, and inserted into DM7 (Zürcher et al., 2013) by LIC. *ALMt9-GFP* was kindly provided by Ulrike Baetz. *pER-rk* and *pPM-rk* (Nelson et al., 2007) were obtained from the ABRC.

Site-directed mutagenesis was performed analogously to PUP14, which was described in the Chapter 3.2.

Suitable amiRs were identified with the WMD web designer (http://wmd.weigelworld.org) and cloned as described (Schwab et al., 2006, and Chapter 3).

Induction

To obtain deregulated expression from the ethanol inducible AlcR/AlcA promoter system (Roslan et al., 2001), embryos and seedlings were treated as described previously in Chapter 3.1.

Protoplast isolation

Protoplast isolation was performed as described in Chapter 3.2 for ETPamir. For subcellular analysis and transport assays, protoplasts were transfected with empty vector (*mER*) and effector vector in a ratio of 1:1. Vacuolar release from protoplasts was done as described (Zhang et al., 2013). Protoplast transport assays were carried out as described in Chapter 3.1.

Onion cell microprojectile bombardment

Epidermal peels of fresh and healthy onions were chosen. Transient onion transformation

was performed as described (Varagona et al., 1992). Transformed tissue was assessed using either a Leica DM6000B light microscope equipped with epifluorescence or by LSCM using a Leica SP2 (Leica Microsystems, Heerbrugg, Switzerland).

Tobacco infiltration and transgene induction

Tobacco leaf infiltration was performed as described (Sparkes et al., 2006). To minimize silencing effects, the *p19* plasmid was coinfiltrated (Voinnet et al., 2003). For induction of the transgenes, 1 mL of 5 % (v/v) EtOH in Eppendorf tubes were placed in each pot next to the plant for vapour induction for 48 hours. Transformed leaves were analysed by LSCM using a Leica SP5 (Leica Microsystems, Heerbrugg, Switzerland).

Picture processing of micrographs

Image processing, particularly maximum intensity projection of confocal image z-stacks in Fig. 1, was done using Imaris (Bitplane, Zurich) provided by the Centre for Microscopy and Image Analysis of the University of Zurich.

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5 General Discussion

GENERAL DISCUSSION

The phytohormone class of cytokinins is known to be involved in such diverse processes as the regulation of meristem size in the shoot (Gordon et al., 2009; Chickarmane et al., 2012; Azizi et al., 2015; Adibi et al., 2016) and root (Dello Ioio et al., 2007; Müller and Sheen, 2008; Carlsbecker et al., 2010; Zhang et al., 2013a), apical dominance (Wickson and Thimann, 1958; Tanaka et al., 2006; Müller et al., 2015), delay of senescence (Richmond and Lang, 1957; Gan and Amasino, 1995; Riefler et al., 2006; Kim et al., 2012; Zwack et al., 2013; Talla et al., 2016), tissue regeneration (Skoog and Miller, 1957; Buechel et al., 2010; Hill and Schaller, 2013), nodulation (Tirichine et al., 2007; Sasaki et al., 2014), and many others. Cytokinin signalling is initiated by ligand binding to its cognate receptor which elicits intracellular phosphorelay signalling via a two-component system that typically ends in the transcriptional activation of cytokinin target genes (Hwang and Sheen, 2001). Due to the myriad of processes controlled by cytokinin, it is self-evident that there are many ongoing efforts to decipher further details in the realm of cytokinin signalling and function. Likewise, this dissertation has aimed at deepening our understanding of cytokinin signalling regulation. In Chapter 2, we have reported the optimization of the cytokinin signalling reporter that led to the design of *TCSn::GFP*. This reporter has become an invaluable tool in the characterization of the *Arabidopsis PURINE PERMEASE* gene family that was the prioritized aim of my thesis. The vascular plant specific family of *PUPs* was chosen as candidates to address the long-standing question of how cytokinin signalling landscapes are established. Within this family, we have focused our main attention on *PUP14*, as described in Chapter 3. By the analysis of *PUP14* knockdown lines, expression studies and transport assays, we could establish that PUP14 functions to shape cytokinin signalling landscapes by withdrawing active cytokinins from the apoplast whereby signalling is attenuated. These findings unravel a mode of signalling control that is without precedent.

Our work on other *PUP* family members, which is the subject of Chapter 4, shows that some *PUPs* function similarly to *PUP14*, whereas others, especially *PUP18*, probably execute tasks that differ from the postulated *PUP14* role. The results obtained for the *PUP* members aside *PUP14* are largely preliminary and make no pretence of completeness. Nevertheless, I will discuss the findings of the entire project in this section and try to put them into the larger context of cytokinin signalling and plant development.

The site of cytokinin perception

While the core cytokinin signalling pathway has been elucidated (Hwang and Sheen, 2001), there was some dispute about the site of cytokinin perception by the cytokinin receptors, AHK2, AHK3, and AHK4. Based on bioinformatics predictions, AHK4 was assumed to reside in the plasma membrane (Inoue et al., 2001; Ueguchi, 2001), and an AHK3-GFP fusion protein was indeed found localized to the plasma membrane in protoplasts (Kim et al., 2006). More recent evidence however suggested that the bulk of *Arabidopsis* and maize AHKs is residing in the ER membrane (Caesar et al., 2011; Lomin et al., 2011;

Wulfetange et al., 2011). Tobacco leaf epidermal cells transfected with *35S::AtAHK3-GFP* showed signal in the ER (Caesar et al., 2011; Wulfetange et al., 2011) and bimolecular fluorescence complementation (BiFC) with *AtAHK2* also revealed localization in the ER network. By membrane fractionation it was shown that AHK3-Myc protein accumulates in the same fraction as ER markers (Wulfetange et al., 2011). For ER-residing AHK receptors, it is likely that their topology allows them to realise phosphotransfer onto the cytosolic AHPs (Punwani et al., 2010), which implicates that the cytokinin-binding CHASE domain faces the ER-lumen. Members of the ISOPENTENYLTRANSFERASE protein family that catalyse the first step in cytokinin biosynthesis are located in the cytosol, mitochondria and plastids (Kakimoto, 2001; Takei et al., 2001; Kasahara et al., 2004), whereas the cytokinin-activating LONELY GUY (LOG) enzymes function in the cytosol and nucleus (Kurakawa et al., 2007; Kuroha et al., 2009). This discrepancy of proposed sites of synthesis and perception raises the question of how the produced cytokinins are transported to the receptors. Our initial working hypothesis therefore insinuated that PUP members function to transport cytoplasmic cytokinin into the ER lumen to the ligand-binding domain of the ER-localized AHKs. However, the localization studies using GFP-tagged PUPs in protoplasts and *in vivo* show that PUPs preferentially locate to the plasma membrane, which precludes ER transport as prime function for the tested PUPs. Furthermore, the expression patterns that we observed for *PUP4*, *PUP11* and most strikingly for *PUP14*, imply that their role conflicts with cytokinin signalling activity. The research that revealed ER-location for AHKs is immaculate in its execution. However, a functional relevance for the endomembrane-localized receptors was not provided, and the possibility of plasma membrane targeting was not excluded by the authors (Lomin et al., 2011).

Predominant subcellular localization and ligand binding do not need to overlap as was described for AUXIN BINDING PROTEIN1 (ABP1)[1] (Jones and Herman, 1993; Tian et al., 1995). Similar to the AHKs, ABP1 was found to locate primarily to the ER, however immunocytochemical analysis, photolabelling experiments and determined pH preferences suggested that binding of auxin by ABP1 does not occur at the ER but rather at the plasma membrane. Analogously, there is a body of evidence inferring that AHK receptors locate to the plasma membrane where they play a role in sensing apoplastic cytokinins, although this conflicts with the determined local pH optimum which lies between pH 6 and pH 7 (Romanov et al., 2006). The abundance of free bases in the apoplast (Jiskrová et al., 2016; Antoniadi et al., *personal communication*, March 2016) which are relevant for cytokinin signalling (Schmitz et al., 1972; Romanov et al., 2006; Lomin et al., 2015) supports the idea of plasma membrane localized receptors.

In many plant species, metabolic cytokinin inactivation occurs primarily via degradation through CYTOKININ OXIDASES (CKXs) (Mok and Mok, 2001). Of the 7 CKX variants encoded in *Arabidopsis*, six have been experimentally (CKX1, CKX2, and CKX3) or by prediction (CKX4, CKX5, and CKX6) associated with the secretory pathway (Werner et al., 2003; Köllmer et al., 2014). In tissue cultures, CKXs have been reported to respond to exogenous cytokinin by increased glycosylation (of the CKX) and secretion (Palmer and Palni, 1987; Motyka et al., 1996; Jones and Schreiber, 1997). Although, it is not clear whether

1 Unfortunately, ABP1 has recently fallen from grace (Gao et al., 2015). This shall not derogate ABP1 as example for the point I am trying to make.

the elevated glycosylation is directly induced by cytokinin or whether the glycosylation is causally linked to increased secretion, the elevated levels of secreted CKX shows that the apoplastic cytokinin concentrations need to be kept in balance. This could provide an effective mode to limit excessive signal induction through plasma membrane AHKs. Indeed, in the bryophyte *Physcomitrella patens*, the heterologous expression of *AtCKX2* affected concentrations of extracellular iP and its riboside (iPR), while the intracellular cytokinins were not significantly altered (von Schwartzenberg et al., 2007). The decrease in extracellular iP and iPR levels were accompanied by drastic phenotypes including abnormal protonema cells and lack of sexual reproduction (von Schwartzenberg et al., 2007), revealing the significance of extracellular cytokinin.

In Chapter 3.1, we provided similar evidence for the role of apoplastic cytokinin sensing; CKX2-mediated cytokinin degradation in the extracellular space drastically reduces signalling in protoplasts. In contrast, cytokinin degradation in the cytosol or ER lumen does not have a pronounced effect, demonstrating that primarily extracellular cytokinins account for the signalling response. Together, our findings and the observations from other studies created an adapted dogma of cytokinin perception and inherently allowed us to make amendments in regards to the hypothesized function of PUPs.

The role of PUP14 in shaping cytokinin signalling domains

The inducible downregulation of *PUP14* in both *amiR* lines, *amiR14_1* and *amiR14_2*, leads to broad ectopic cytokinin signalling as revealed by the cytokinin signalling output reporter *TCSn::GFP* (Zürcher et al., 2013). The shoot phenotype that is characterized by increased branching, phyllotaxis aberrations and a larger apical meristem, and the seedling's short root phenotype are indicative of increased cytokinin output (Mason et al., 2005; Miyawaki et al., 2006; Argyros et al., 2008; Ishida et al., 2008; Bartrina et al., 2011; Skylar and Wu, 2011). These findings suggest that PUP14 functions to limit undesired cytokinin signalling output. Because PUP14 at the plasma membrane acts as an importer of tZ, the observed *amiR* phenotype is in line with the stated significance of apoplastic cytokinin in eliciting signalling. Moreover, the complementarity of *PUP14* and *TCSn::GFP* expression underpins the inhibitory role of the transporter on signalling. What remains unclear is how in turn the pattern of *PUP14* expression is established. The nearby solution would be that cytokinin signalling directly interferes with *PUP14* expression or stability. Interestingly, a microarray meta-analysis indeed found *PUP14* to be downregulated by cytokinin (Bhargava et al., 2013) suggesting mutual regulation. To identify, which of the two players, PUP14 or cytokinin signalling are established first, one can argue that enzymes involved in cytokinin production or activation are potentially specifically expressed to establish a prepattern of cytokinin distribution. As a matter of fact, mRNA of *LOG3* whose gene product is involved in cytokinin activation (Kurakawa et al., 2007), is found in the provasculature of embryos (De Rybel et al., 2014), concurring with cytokinin signalling output. However, this colocalization of cytokinin activation and signalling is hardly repeated in other contexts; as we have shown in the introduction (Chapter 1.1, Fig.3), neither the expression sites of *LOG* family members (Kuroha et al., 2009), nor the ones of *IPT* family members (Miyawaki et al., 2004),

coincide with cytokinin signalling activity. For instance in the shoot apical meristem (SAM), LOG4 localizes to the L1 layer, whereas cytokinin signalling is located in the stem cell niche and organizing centre (Gordon et al., 2009; Chickarmane et al., 2012). In cases where cytokinin production and signalling do not overlap, PUP14 might contribute to translocation of cytokinins to defined perception sites. Consistently, we found that TCSn::GFP signal is broadened in the SAM of *PUP14* knockdown lines. However, in contrast to TCSn::GFP expansion in the embryo, the SAM does not show upregulated signalling in the entire organ. This indicates that in the SAM environment additional components are regulating signalling. Indeed, *AHK4* receptor expression localizes almost congruently with TCSn::GFP and application of exogenous cytokinin only enlarges the response to *AHK4*-expressing areas (Gordon et al., 2009), showing that also the competence of ligand sensing can be a limiting factor in cytokinin landscapes. Such alternative determinants that contribute to the shaping of cytokinin landscapes might explain why overexpression of *PUP14* (and other *PUPs*) did not alter signalling as successfully as did downregulation. Intuitively, the expected outcome of *PUP14* overexpression is quenching of the endogenous signal. Although we could observe this in several instances, many embryos were not affected in the endogenous, provasculature signal. Presumably, the plant has a high interest in maintaining this local cytokinin maximum, and therefore ensures that several additional mechanisms provide robustness to this domain. Incidentally, not only the previously mentioned *LOG3* but also *AHK4* displays high mRNA levels in the provasculature (Mähönen et al., 2000), thereby enhancing production and signalling, respectively. In summary, rather than defining areas of signalling activities, the role of *PUP14* lies in defining areas of trespassing, where cytokinin signalling is strictly undesired.

Downstream cytokinin metabolism

We have shown transport capacity of PUP14 and other PUP family members, however, the fate of the imported tZ is not clear. The most prominently observed paths for intracellualr cytokinins are glucosylation or oxidative cleavage by CKX (Martin et al., 1999; Werner et al., 2003; Hou et al., 2004; Sakakibara, 2006). In detached leaves of *Populus alba*, the addition of exogenous cytokinin leads to the accumulation of *O*-glucoside conjugates (Duke et al., 1979). Because glucosyl-conjugates are usually inactive in bioassays (Haberer and Kieber, 2002), this implies that excess cytokinin is metabolised further to maintain the low intracellular level of the active free bases. Similarly, feeding experiments in *Arabidopsis* root protoplasts performed by Ioanna Antoniadi have alluded to increasing intracellular levels of the respective ribose monophosphate after addition of tZ or cZ (Ioanna Antoniadi, Ondrej Novak, Thomas Vain, Markéta Pernisová, Radim Simersky, Václav Mik, Lenka Plačková, Jan Hejátko, Stephanie Roberts, Karel Doležal, Karin Ljung, Colin Turnbull, unpublished). The conversion of the cytokinin free base into the nucleotide is catalysed by ADENINE PHOSPHORIBOSYL TRANSFERASE1 (APT1) (Moffatt et al., 1991; Zhang et al., 2013b). APT1 is predicted to locate to plastids and its activity was found to be relevant for normal development (Moffatt and Somerville, 1988; Zhang et al., 2013b). The cytokinin-related growth defects in overexpressors of the cytosolic *CKX7* (Köllmer et al.,

2014) further illustrate the importance of balanced intracellular cytokinin homeostasis; *CKX7* overexpression caused a short primary root, loss of lateral roots and defects in root vasculature development. This is in contrast to the elongated primary root and increased root branching observed during *CKX1* or *CKX2* overexpression, which have similar reductions in total cytokinin (Werner et al., 2003; Köllmer et al., 2014). The conflicting consequences in phenotypes elicited by intra- versus extracellular *CKX* variants show that rather subcellular distribution than absolute levels of cytokinins determine the developmental programme.

In addition to its function as signalling molecule, cytokinin can be used as a precursor for adenine, adenosine or even adenosine monophosphate since the action of CKX cleaves its substrates into the nucleobase, nucleoside or nucleotide, and the respective cytokinin N^6- side chain (Brownlee et al., 1975; Armstrong, 1994; Galuszka et al., 2007). Cytokinin feeding experiments in *Physcomitrella patens* showed that only a fraction of administered radiolabel is found again in cytokinin metabolites; purine-like products from CKX-mediated cytokinin degradation were suggested to end up in macromolecules including RNA (von Schwartzenberg et al., 2007). Because of the vital role of adenine nucleotides as energy currency (Haferkamp et al., 2011), the spectrum of cytokinin functions should not be exclusively associated with signalling but rather acknowledge the potential of its metabolites.

In our assays, we primarily focused on tZ, the most abundant cytokinin in many species including *Arabidopsis*. The overall effect of the different cytokinins is by and large the same and also defines the individual molecule as cytokinin, however there are differences between cytokinins in their bioactivities, and sometimes effects. This can be exemplified by tobacco seedlings treated with iP, dihydrozeatin or tZ in which iP and tZ are more effective in inhibiting primary root growth than dihydrozeatin (Lexa et al., 2003).

Apart from merely differing in their inherent biological activities, differences in effectiveness can be attributed to substrate preferences of cytokinin metabolising enzymes. The levels of cZ, cZ 9-glucoside (cZ9G), and iP 9-glucoside (iP9G) are specifically more reduced by *CKX7* overexpression than by *CKX1* or *CKX2* overexpression (Köllmer et al., 2014), indicating that cZ, cZ9G and iP9G are the preferred substrates for CKX7. Nonetheless, the root vasculature phenotype of *CKX7* overexpression is more efficiently rescued by exogenous application of tZ than of cZ (Köllmer et al., 2014). In line with differing substrate affinities, iP and iPR have been found to be preferred substrates for most CKXs (Armstrong, 1994), whereas the aromatic cytokinin BA is almost resistant to oxidative cleavage by CKX (Galuszka et al., 2007). Similarly, *O*- and *N*-glycosylation do not occur equally frequent on the different cytokinins (Veach et al., 2003; Hou et al., 2004).

Despite the evidence favouring apoplastic signalling initiation, it is obvious that the levels of intracellular cytokinins are under equally tight regulation. The enzymes that determine the metabolic fates of cytokinins, amongst which are the *O*- and *N*-glycosyltransferases, are required for normal cytokinin distribution and partitioning (Jin et al., 2013; Wang et al., 2013). However, the absence of a phenotype for the respective mutants demonstrates that the plant tolerates or compensates deviations from regular intracellular cytokinin profiles under normal growth conditions.

The potential roles of other PUP members in plants

Besides *PUP14* expression, *PUP4* and to a limited extent *PUP11* expression also displayed complementarity to cytokinin signalling. Based on the largely conserved tZ import capacity among PUPs, it seems that PUP4 and potentially other, less expressed family members adopt similar roles as PUP14 in shaping substrate distribution. Evidently, the gene duplications that have resulted in the high number of *PUP* family members are likely to have brought about gene sub- and neofunctionalization (Jelesko, 2012). In analogy to the different substrate specificities of metabolic enzymes, we can therefore expect that substrate affinities and specificities differ between PUP family members. Additionally, expression of the less abundantly found members is likely to be induced under specific circumstances. In agreement with this prediction, available microarray data shows that *PUP10* is induced upon senescence (Hruz et al., 2008), a process known to be regulated by cytokinin. We could recapitulate the observation of increasing *PUP10* transcript in dark-induced senescence by qRT-PCR (not shown). Such highly context-dependent expression is more difficult to identify, but gives insights into the specific role of a given *PUP*. The already ample knowledge of cytokinin's sites of actions allows future research to directly look at *PUPs* within these contexts.

In accordance with possible neofunctionalization, *PUP18* shows an expression pattern that is deviating from the cytokinin-complementary pattern observed for *PUP14* and *PUP4*. PUP18's consistent localization in the L1 layer is hinting to a role in communication with the environment. PUP18 only moderately improved tZ uptake into isolated protoplasts. Thus, it is likely to exert a different function than confining cytokinin signalling domains. Nonetheless, the fact that we found some ectopic *TCSn::GFP* signalling after *PUP18* overexpression suggests that its function is related to cytokinin signalling. But the precise role still needs to be elaborated.

Comparing cytokinin to auxin transport

Recently, the interplay between auxin and cytokinin has been referred to as Yin and Yang (Schaller et al., 2015), alluding to their complementary rather than often claimed opposite roles in which only their concerted action allows the accomplishment of a task. In spite of the beauty of this "together as one" concept, I tend to think of the reciprocity of auxin and cytokinin as a love-hate relationship; the anthropomorphised synonym of an elderly couple that always fights, but never parts, the two being equally friends and enemies - one moment they get along (Leibfried et al., 2005; Zhao et al., 2010), next moment they stab each other's back (Müller and Sheen, 2008; Marhavý et al., 2013, 2014). Because of this continuous latent rivalry, it is only natural that I want to take the opportunity to rapidly highlight the conspicuous differences between the roles of PUP-mediated cytokinin and PIN-mediated auxin transport which represents a (previously unique) paradigm of signalling control through hormonal distribution.

Auxin, a ubiquitous phytohormone controlling essentially all aspects of plant development (Benjamins and Scheres, 2008; Bennett, 2015), is distributed from cell to cell via an intricate transport system known as polar auxin transport (PAT) (Goldsmith, 1977). The

chemiosmotic theory had predicted that protonated auxin in the apoplast (pH ~ 5.5) could enter cells freely, whereas carriers would be required to mediate efflux of cytosolic (pH ~ 7), deprotonated and therefore negatively charged auxin. Because auxin transport was observed to be strictly directional, the postulated carriers needed to be asymmetrically localized (Rubery and Sheldrake, 1974; Raven, 1975). The following identification of the polarly localized PINs validated the predictions of the chemiosmotic theory (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998). Because of the asymmetric distribution of PINs and the serial connection of cells, PIN-mediated transport realises an auxin-flux that cumulates in the establishment of local auxin maxima (Friml et al., 2002a, b; Benková et al., 2003; Friml et al., 2003; Žádniková et al., 2010; Band et al., 2012). This is fundamentally different from how we found PUP14 to function:

- PINs mediate export of auxin while PUP14 proteins act as importer entailing furthermore that they need not be polarly localized. In order to establish a gradient across several cells, placing an importer asymmetrically is futile as long as the corresponding exporters are not adequately located on the opposing end. Otherwise, the undirected efflux would dissipate the gradient.
- Expression of PUP14 in a particular cell leads to sequestration of cytokinin inside of the cell. Once inside, the hormone is trapped in this dead end rather than engaging in a directional flow as does auxin.
- The signalling mechanisms employed by the auxin and cytokinin hormones rely on intra- versus the described extracellular receptor binding, respectively (Mockaitis and Estelle, 2008). Therefore, cells adjacent to *PIN*-expressing cells, particularly at an auxin maximum, turn on auxin signalling. In contrast, direct proximity to *PUP14*-expressing cells should be correlated with low cytokinin signalling because of the low apoplastic ligand concentration.

This last prediction is based on the observation that often the sum of *PUP14* expression and cytokinin signalling areas does not constitute an entire organ. Rather there are cells between the two zones that may act as buffers. However, given that the apoplast provides a larger space than could possibly be covered by the PUP14 action radius, it is improbable that import could drain significant amounts of cytokinin from neighbouring cells.
The direct comparison of the mechanisms controlling auxin and cytokinin spatiotemporal distribution, clearly shows that the two systems are fundamentally different. Revisiting my initially drawn picture of the elderly fighting couple, this might be filed under “irreconcilable differences”.

General considerations and future perspectives

The use of the TCSn::GFP reporter was instrumental in determining the candidates for a cytokinin transport system. Although we did encounter silencing effects, the overall robustness of the sensor allowed us to detect alterations in signalling activities faithfully. On the downside, the use of the cytokinin reporter constitutes a biased approach as PUP members mediating transport of other compounds are excluded. Although even

the overexpression of *PUP14* produced inconsistent effects, preference of alternative substrates might explain why the overexpression of *PUP4*, *PUP11*, *PUP15* or *PUP23* did not affect TCSn::GFP at all. The other particularity in our PUP screening besides TCSn::GFP, was the use of the ethanol-inducible system (Roslan et al., 2001). It allows the assessment of a gene's function in specific developmental windows. Because loss of gene functions might be deleterious, the system provides a useful workaround to analyse also essential genes by inducible down- or upregulation. We have, however, encountered some flaws in the use of inducible transgenes, the most obvious being that it doubles the number of samples to analyse, as non-inducing and inducing treatments are run in parallel. In regards to the efficiency of induction, we have noted that the yielded pattern of overexpression was never uniform. This can be attributed to posttranscriptional regulation but also to inefficient induction. The fact that we often detected expression of GFP-tagged *PUP* variants on the surface of the embryo could be taken as indicator for low permeability of the ethanol. Induction of the embryos was performed by placing cut siliques into ethanol-containing medium. The embryos were therefore still within the protective environment provided by the seed, which in turn was residing in the marginally manipulated silique. The treatment might not constitute an appropriate approach to induce expression in all cells of the enfolded embryo. For the amiRNA however, we could find that the ectopic expression in induced embryos was homogenously distributed throughout the embryo, implying that downregulation of *PUP14* occurred in all relevant cells. There is a simple rationale to explain why ethanol induction has amounted to different levels in *PUP* and *amiR* expression; it is known that miRNAs can act systemically allowing them to exert their function non-cell autonomously (Yoo et al., 2004).

To resolve the issue of heterogeneous induction associated with ethanol treatment, one could seek to drive expression of transgenes by endogenous, context-specific promoters. Rather than relying on artificial induction, the plant itself then governs gene regulation according to the spatial and temporal regulatory features imposed by the promoter. Currently, a web-based tool that compiles spatio-temporal transcriptome data of the *Arabidopsis* embryo is being set up (www.albertodb.org; Dolf Weijers, *personal communication*, March 2016), which may be used to rapidly identify appropriate candidate promoters to drive cell- or stage-specific expression.

Inherently, the promoters of these early specifying genes would allow lineage-specific expression of a transgene of interest. In our case, *PUP14* expression could be specifically induced in the provasculature of the embryo, to study whether cytokinin signalling is efficiently quenched. The lens-shaped cell that is derived from the hypophysis around the globular stage constitutes another interesting locale for ectopic *PUP14* expression. The hypophysis shows early cytokinin signalling output, which after its division into apical cell and basal cell is specifically retained in the apical lens-shaped cell but not in the basal cell. These precisely regulated signalling activities are a result of an intimate interplay of auxin and cytokinin feedback networks, disturbance of which causes severe morphological defects in the embryonic root stem cell system (Müller and Sheen, 2008). Instead of allowing ectopic cytokinin signalling in the basal cell, as has been done already

(Müller and Sheen, 2008), *PUP14* expression in the lens-shaped cell should cause loss of signalling that is expected to interfere with regular development. Naturally, these precise expression patterns in the provasculature or lens-shaped cells could be achieved by the use of the *TCSn* promoter. Although it is a synthetic sequence, it integrates the *cis*-regulatory elements of the endogenous promoters of type-A *ARRs* (Sakai et al., 2000; Imamura et al., 2003; Müller and Sheen, 2008; Zürcher et al., 2013). It must be taken into account that the expression through *TCSn* is dependent on cytokinin, therefore the induction of factors that inhibit signalling (such as PUP14) creates a feedback loop where the presence of the inhibitor dampens its own expression. To avoid such a scenario, alternative promoters that do not depend on cytokinin should be devised. The *LOG3* or *PIN7* promoters might be tried to achieve provasculature and lens-shaped cell specific expression, respectively (De Rybel et al., 2014; Friml et al., 2003). However, it is questionable whether they are entirely independent of cytokinin signalling.
In order to identify functions of the many *PUP* members, analysis of single mutants might not portray a suitable practice. Due to the probable redundancies between family members, the creation of higher order mutants is key in elucidating their gene functions. The assessment of multiple PUP knockouts or knockdown should therefore be considered for future research. We have used RNA interference (RNAi) to reveal the function of *PUPs* in development. Although over the past decade RNAi-based techniques have become the method of choice to determine gene function, the silver bullet for gene charcterization still are DNA mutations. With the recent advent of the highly praised CRISPR/Cas9 technique that allows the targeted mutation of a gene of interest by RNA-guided target strand cleavage (Jinek et al., 2012), RNAi-based approaches are expected to become less popular (Barrangou et al., 2015; Unniyampurath et al., 2016). Its practicability in plants makes the CRISPR/Cas9 system a white hope for assessing gene functions even in crop species (Liu et al., 2016). Future research on *PUP* family members therefore could profit from the newly implemented technique to create single and multiple knockouts to address *PUP* functions in plant development.

The plant's body plan is laid down as early as during embryogenesis when apical-basal and radial axes are defined (Jürgens, 2001; Friml et al., 2003). The embryo therefore represents a basic, juvenile version of the plant. Because the embryo harbours the precursors of the cells making up the entire plant, embryonic deviation from the default developmental plan are probable to lead to abortion or to cause irregularities throughout the plant's life span. Cytokinin signalling occurs already during this critical developmental stage and because of the many implications this infers, we have focused a large fraction of our analyses on the embryo. However, the range of cytokinin actions is much broader than determining proper embryo formation. Amongst others, cytokinin signalling is indispensable for the formation of the vasculature, which is reflected in the protoxylem-only phenotype of (multiple) knockouts in signalling components (Mähönen et al., 2000; Nishimura et al., 2004; Hutchison et al., 2006; Kuroha et al., 2006; Argyros et al., 2008). The vascular patterns are established in the embryo (Scheres et al., 1994), and the defects in the mentioned mutants should be

attributed at least in part to the early defects in cytokinin signalling. Despite the prominence of the cytokinin-dependent vasculature phenotype, we have not followed the development of the vasculature in our knockdown lines in detail. As we have noticed that root growth in the *PUP14* knockdown lines is inhibited, it might well be that vasculature development is compromised. It needs to be taken into account however that–although they all display a short-root phenotype–cytokinin signalling is increased in *PUP14* knockdown, while it is decreased in *wol* or multiple type-B *arr* and *ahp* knockouts (Hutchison et al., 2006; Mähönen et al., 2006; Argyros et al., 2008). This apparent paradox is a paradigm of the biphasic response that has been described for cytokinin-mediated root inhibition (Ferreira and Kieber, 2005). To determine the underlying cause for stunted root growth, in the *amiR* lines, histological assessment of root sections and the combination of the mutant with lineage-specific markers could be carried out in the future. Similarly, the observations of aberrant phyllotaxis, increased meristem size or elevated branching can be scrutinized to give deeper insights into the spatiotemporal process of cytokinin regulation in these contexts.

While cytokinins do occur already in bryophytes (von Schwartzenberg et al., 2007), the *PUP* gene family is specific to vascular plants (Hildreth et al., 2011), which might reflect their importance in establishing the complex patterns of higher plants. Because of the large number of variants in *Arabidopsis*, it is difficult to identify the original function of *PUPs* in this species. Likewise, several *PUP* members have been found for rice, tobacco, *Vitis vinifera*, *Populus*, corn and other monocot and dicot species (Jelesko, 2012). The most basal plant genome in which *PUP*-like sequences could be identified is *Selaginella moellendorffii*, which already harbours three paralogous groups of *PUP*-like transporters with a total of 10 homologues (Hildreth et al., 2011; Jelesko, 2012). The analysis of these homologues could determine whether the role in defining cytokinin signalling landscapes had already been established in *PUPs* of this early land plant.

Altogether, what we know about the role of *PUPs* is probably only little, and what we understand is even less. Hopefully, ongoing and future research will uncover the functions of these genes, potentially helping towards a better understanding of plant development.

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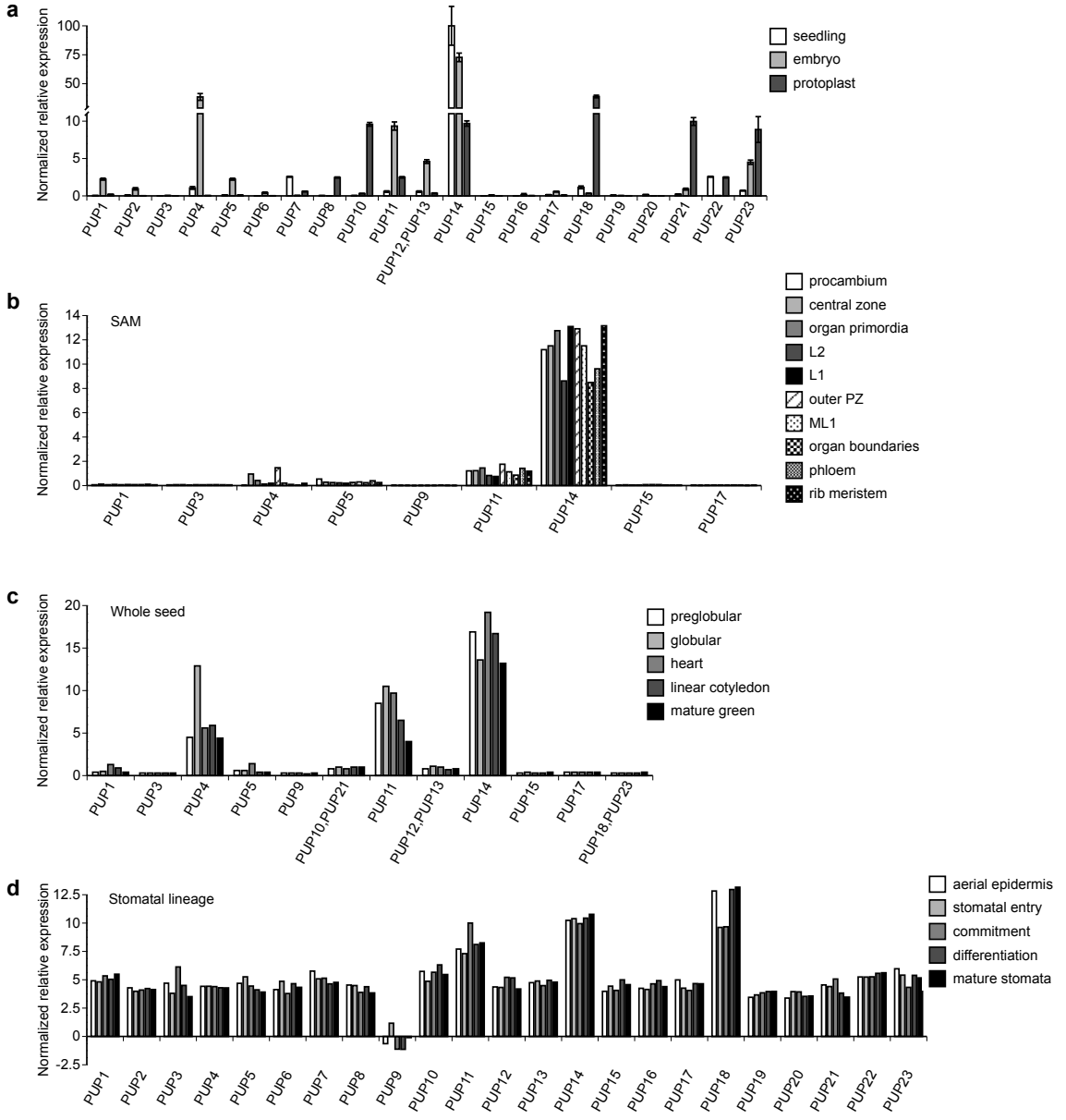
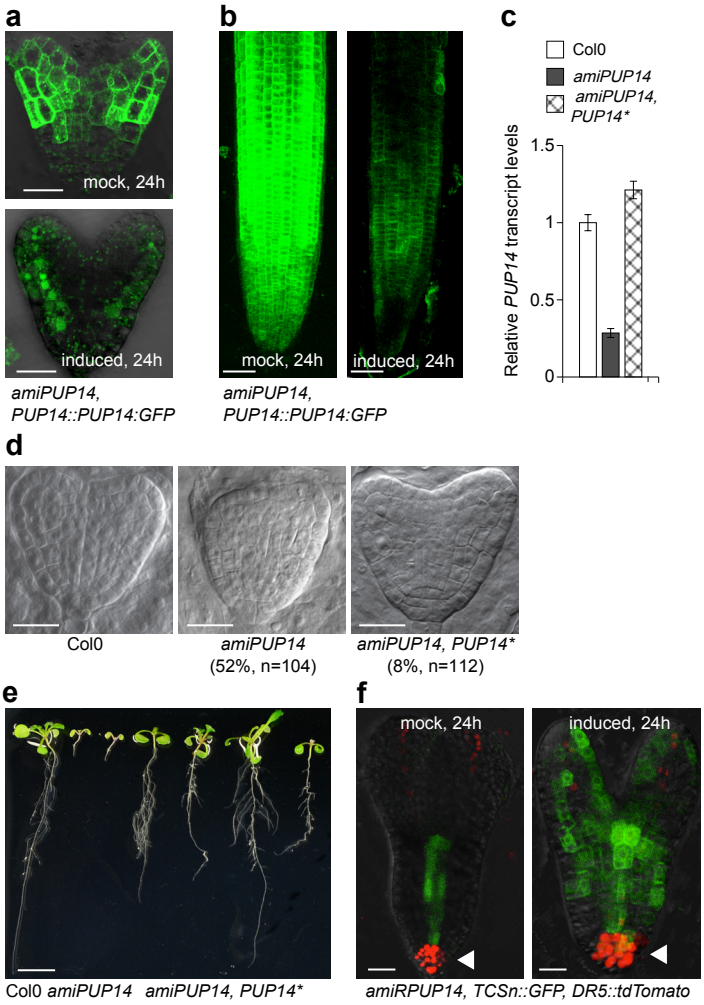
Appendix

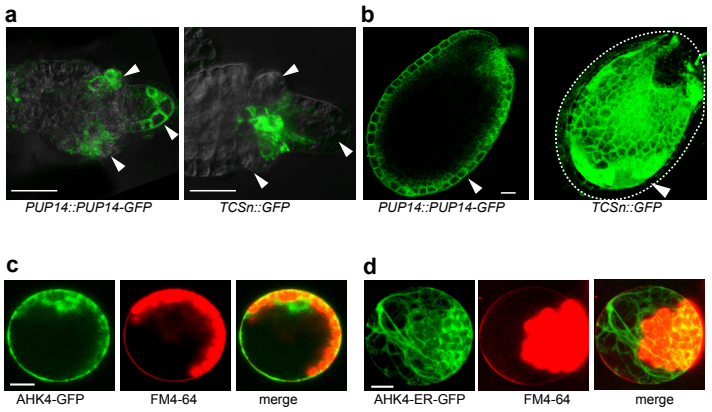
APPENDIX A1



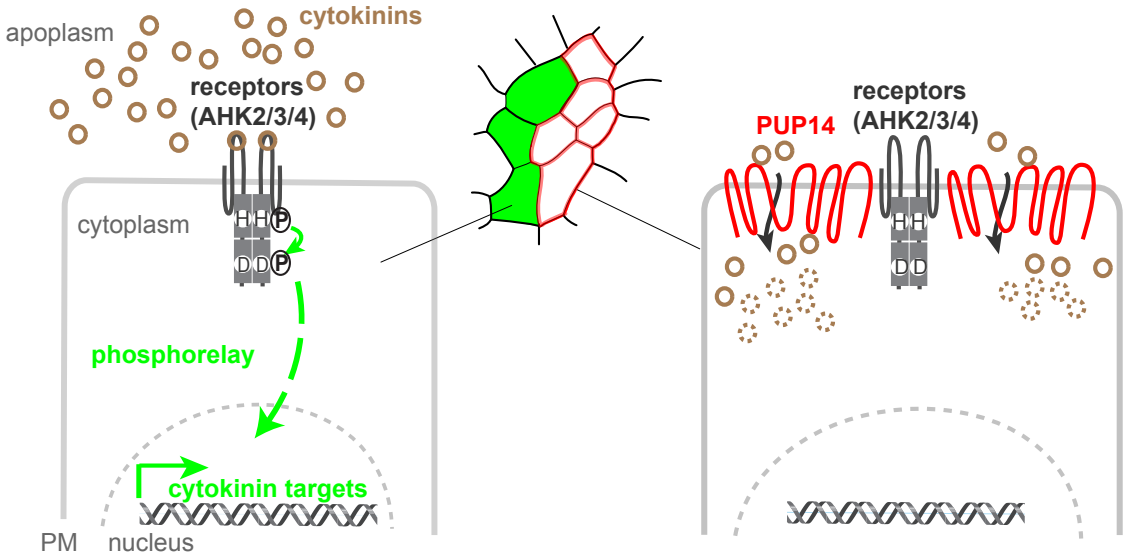
Extended Data Figure 1 | Conspicuous *PUP14* expression in different developmental contexts
a, Transcription profile of *PUP* family members in seedlings, embryos and mesophyll protoplasts determined by qRT-PCR. b, Cell type-specific ATH1-based microarray dataset of *PUPs* in the SAM. L1 = layer 1 in SAM, L2 = layer 2, ML1 = layer 1 in meristem and in differentiating organs, PZ = peripheral zone (Yadav et al., 2014). c, ATH1 based microarray dataset of *PUPs* during seed development (Belmonte et al., 2013). d, Stage-specific RNA-seq dataset of *PUPs* in stomatal lineage (Adrian et al., 2015). *PUP* AGI identifying numbers: *PUP1*, AT1G28230; *PUP2*, AT2G33750; *PUP3*, AT1G28220; *PUP4*, AT1G30840; *PUP5*, AT2G24220; *PUP6*, AT4G18190; *PUP7*, AT4G18197; *PUP8*, AT4G18195; *PUP9*, AT1G18220; *PUP10*, AT4G18210; *PUP11*, AT1G44750; *PUP12*, AT5G41160; *PUP13*, AT4G08700; *PUP14*, AT1G19770; *PUP15*, AT1G75470; *PUP16*, AT1G09860; *PUP17*, AT1G57943; *PUP18*, AT1G57990; *PUP19*, AT1G47603; *PUP20*, AT1G47590; *PUP21*, AT4G18220; *PUP22*, AT4G18205; *PUP23*, AT1G57980.



Extended Data Figure 2 | *amiRPUP14*-induced phenotypes are specific to *PUP14*
a,b, Strong decrease of PUP14::PUP14-GFP levels in (a) heart-stage embryos and the (b) seedling root tip after 24 h of *amiRPUP14* induction compared to mock, n=6. c, Relative *PUP14* and *PUP14** transcript levels in *PUP14**-complemented seedlings compared to wild type and amiRPUP14, all ethanol-treated, error bars denote s.e.m. d, *amiRPUP14*-induced morphological defects (61% affected embryos, n=88) in cotyledons and root meristem are complemented in *PUP14R** embryos (9% affected embryos, n=112). e, Root growth is restored in *PUP14**-complemented seedlings. f, *amiRPUP14* specifically affects cytokinin but not auxin response, a shown by unchanged DR5::tdTomato (Lituiev et al., 2013) expression in *amiRPUP14*-induced embryos (arrowheads). Scale bars (a,d,f) 20 µm, (b) 50 µm, (d) 1cm.



Extended Data Figure 3 | *PUP14* expression and AHK4 subcellular localization
a,b, PUP14::PUP14:GFP and TCSn::GFP expression in (a), the developing ovule primordium and (b) the seed. Dotted lines delimit the seed coat. c, AHK4-GFP in mesophyll protoplasts (left), co-stained with FM4-64 dye to mark the plasma membrane (middle) with overlay on the right. d, *AHK4-ER-GFP* is an *AHK4* variant with the ER-retention signal HDEL. No plasma membrane signal detected with AHK4-ER-GFP. Arrowheads indicate peak PUP14 expression and low TCSn::GFP. Scale bars (a,c,d) 10 µm, (b) 20 µm.



Extended Data Figure 4 | Model of *PUP14* function in cytokinin signalling
PUP14 (red) causes the translocation of apoplastic cytokinins to the cytosol, where they become converted to inactive forms (dotted circles). This results in reduced binding to plasma membrane-localized cytokinin receptors, and consequently reduced signalling activation (green denotes cytokinin signalling activity). PM: plasma membrane.

Target gene	Orientation *	Sequence 5‘ - 3‘
eIF4a	F	TCATAGATCTGGTCCTTGAAACC
	R	GGCAGTCTCTTCGTGCTGAC
ARR5	F	GGTTGGATTTGAGGATCTGAAG
	R	TCCAGTCATCCCAGGCATAG
ARR6	F	TTGCCTCGTATTGATAGATGTCTT
	R	CCGAGAGTTTTACCGGCTTC
ARR7	F	AGATTAAGGAATCTTCAGCATTCAG
	R	CTGCTAGCTTCACCGGTTTC
mGFP	F	TCAAGGACGACGGGAACTAC
	R	ATCCTGTTGACGAGGGTGTC
PUP1	F	TGTTTCCGGGAGAAGTTTCA
	R	CGGATTTAAACTCGCCGTAG
PUP2	F	TCTGTGCATCGTCTCTGGTC
	R	TCTCCTGGAAGCAAATGACG
PUP3	F	AATACCCGAGACGAGAGACG
	R	CGTCTCTCGTCTCGGGTATT
PUP4	F	ACCGGAGGTATCTGCATGAC
	R	CACTCCACCAAACACGTCAC
PUP5	F	TGCAGTCACGTTTCAACTGG
	R	TGACTGTGGATGCCAGAAAC
PUP6	F	TGCCTGTTCTTGCTGTTGTC
	R	TCTTGGTCTTCTTCTGGCTTTC
PUP7	F	TTTAGCTATCTGCGGCTTCC
	R	GTGTGACCTTCCTCAACAGG
PUP8	F	GTCGTGGGACTGATCTTTGAG
	R	GCAATCCCACAGCAGTTATG
PUP10	F	ACCCACCAGAAGCAGAAGAG
	R	GTAAACTGCGGGACAGCATC
PUP11	F	TCGACGTATTCGCTCATTTG
	R	GCGGAGAACGACAAGAGAAC
PUP12/13	F	AGGTTAAGATGGTGGCGATG
	R	TGAGCTTCTCGTGCTCTTTG
PUP14	F	TCTGTTTCGAGCGTGTTGTC
	R	GCGCTTAAGACGGCAGTAAC
PUP15	F	GCAGCTGCTCTTAGCGTCTC
	R	TTGTGGATTGGTCATCATCG
PUP16	F	GTCCGGTTTATTCGCTGATG
	R	AGCACCTCTTCTCTGCCAAC
PUP17	F	GGCCTAGAATTGGTGCTTTG
	R	TTTGGTTAAGTTCCGCCATC
PUP18	F	TGCTTTATGTTTCGGGTGTG
	R	CAAAGCCACAAGTGGTGAAG
PUP19	F	CTGGTAGCTGGGATTCTTGG
	R	AGTTGTTTGGCTTTGGCTTG
PUP20	F	TTTAGGGCTTGTGGGTCTTG
	R	GCTCCTCCCTTAAACCATCC
PUP21	F	TTGCACAGGACTGATCTTCG
	R	TGACAGCCAGGATAGGAACC
PUP22	F	ATCTTGACTTTGGCCTCAGC
	R	GCAGTCCCACAGCAGTTATG
PUP23	F	TGTGTGCTTCACCACTTATGG
	R	AGCACCAATTCTAGGCCAAC

* F = forward; R = reverse

Extended Data Table 1 | qRT-PCR primer sequences used in this study.

Name	Purpose	Parent vector	Selection (bacteria/plants)	Insert		
				Primer name	Sequence 5‘-3‘	Template
35S>ALC>amiRPUP14_1	Ethanol-inducible binary vector with amiR specific for *PUP14* (variant 1)	DM7-LIC	Kan/Kan	LIC-OLIGO_A	tagttggaatgggttcgaaCGACGTTGTAAAACGACGG-CCAG	pRS300
				LIC-OLIGO_B	ttatggagttgggttcgaaCTCGGAATTAACCCT-CACTAAAGG	
				amiR_14_1_I	gaTTATTTGCACAAAGTGTTCTGtctctcttttgtattcc	
				amiR_14_1_II	gaCAGAACACTTTGTGCAAATAAtcaaagagaatcaatga	
				amiR_14_1_III	gaCAAAACACTTTGTCCAAATATtcacaggtcgtgatatg	
				amiR_14_1_IV	gaATATTTGGACAAAGTGTTTTGtctacatatatattcct	
35S>ALC>amiRPUP14_2	Ethanol-inducible binary vector with amiR specific for *PUP14* (variant 2)	DM7-LIC	Kan/Kan	LIC-OLIGO_A	tagttggaatgggttcgaaCGACGTTGTAAAACGACGG-CCAG	pRS300
				LIC-OLIGO_B	ttatggagttgggttcgaaCTCGGAATTAACCCT-CACTAAAGG	
				amiR_PUP14_2_I	gaTGTTGATAGGTATTTGCACGAtctctctttgtattcc	
				amiR_PUP14_2_II	gaTCGTGCAAATACCTATCAACAtcaaagagaatcaatga	
				amiR_PUP14_2_III	gaTCATGCAAATACCAATCAACTtcacaggtcgtgatatg	
				amiR_PUP14_2_IV	gaAGTTGATTGGTATTTGCATGAtctacatatatattcct	
35S>ALC>AHK3	Ethanol-inducible binary vector (3)	DM7-LIC	Kan/Kan	AHK3_LIC_f	tagttggaatgggttcgaaTGAGTCTGTTCCATGTGCTAG	Col0 genomic
				AHK3_LIC_r	ttatggagttgggttcgaaTGATTCTGTATCTGAAGGCG-AATTG	
PUP14::PUP14-GFP	Reporter	pCB302 LIC GFP	Kan/Basta	PUP14_LICF-F	tagttggaatgggttcgaGCTTCTGCAGTGAAA-GATGTGTT	Col0 genomic
				PUP14_LIC_ GFP302_R	tattggagttgggttcgaaTAAGCCATACGATTGTCTT-TGTG	
hbt::PUP14-GFP; 35S::PUP14	Protoplast expression vector; binary vector for expression in microsomes	*hbt::LIC-GFP*; pPLV26 (33)	Amp; Kan/Kan	PUP14_LIC_F	tagttggaatgggttcgaATCCATGGCTCAGAATCAA-CAAC	Col0 genomic
				PUP14_LIC_R	ttatggagttgggttcgaaATAAGCCATACGATTGTCTT-TGTG	
PUP14 pCB302	*PUP14* genomic region in binary vector	pCB302LIC	Kan/Basta	PUP14_LICF-F	tagttggaatgggttcgaGCTTCTGCAGTGAAA-GATGTGTT	Col0 genomic
				PUP14_ LIC3prime_R	ttatggagttgggttcgaaGCACACTTCCAAACATTTTCA	
*PUP14**	*amiRPUP14_2*-resistant *PUP14* in binary vector	*PUP14 pCB302*	Kan/Basta	amiR14_2R* F	CTCTGTTTCTTTTTGCAGAACAATTTGTCCA-GATTCCAATAAATA	*PUP14 pCB302*
				amiR14_2R* R	GGTTGAAGAATCACGCTCGATATTTATTGG-AATCTGGACAAATTG	
35S::PUP1	Binary vector for expression in microsomes	pPLV26 (33)	Kan/Kan	PUP1_LIC_F	tagttggaatgggttcgaa ACAGCAAGCAGCAAGAAGAA	Col0 genomic
				PUP1_LIC_R	ttatggagttgggttcgaa AGCAACATAATCACTAACAGG-AAG	
hbt::CKX2-HA; hbt::CKX2-GFP	Protoplast expression vector	*hbt::LIC-HA hbt::LIC-GFP*	Amp	CKX2_LIC_f	tagttggaatgggttcgaaTAAACAAATGGCTAATCTT-CGTT	Col0 genomic
				CKX2_LIC_r	GATGTCTTGCCCTGGAGATAACA	
hbt::ΔSP-CKX2-HA; hbt::ΔSP-CKX2-GFP	Protoplast expression vector	*hbt::LIC-HA hbt::LIC-GFP*	Amp	CKX2ΔSP_LIC_f	tagttggaatgggttcgaATGATTAAAATTGATT-TACCTAAATCCC	Col0 genomic
				CKX2_LIC_r	GATGTCTTGCCCTGGAGATAACA	
hbt::CKX2-ER-HA; hbt::CKX2-ER-GFP	Protoplast expression vector	*hbt::LIC-HA hbt::LIC-GFP*	Amp	CKX2_LIC_f	tagttggaatgggttcgaaTAAACAAATGGCTAATCTT-CGTT	Col0 genomic
				CKX2 HDEL_LIC_r	ttatggagttgggttcgaaAAGCTCATCATGGATGTCTTG-CCCTGGAGATAACA	
hbt::CKX7-HA; hbt::CKX7-GFP	Protoplast expression vector	*hbt::LIC-HA hbt::LIC-GFP*	Amp	CKX7_LIC_f	tagttggaatgggttcgaaCACACACACCAAAATGATAGCT	Col0 genomic
				CKX7_LIC_r	AAGAGACCTATTGAAAATCTTTTGACC	
hbt::AHK4-GFP	Protoplast expression vector	*hbt::LIC-GFP*	Amp	AHK4_LIC_f	tagttggaatgggttcgaaTTGAAGTGATGAGAAGA-GATTTTGTG	Col0 genomic
				AHK4_LIC_r	ttatggagttgggttcgaaCGACGAAGGTGAGATAGGAT-TAGG	
hbt::AHK4-HDEL-GFP	Protoplast expression vector	*hbt::LIC-GFP*	Amp	AHK4_LIC_f	tagttggaatgggttcgaaTTGAAGTGATGAGAAGA-GATTTTGTG	Col0 genomic
				AHK4HDEL_LIC_r	ttatggagttgggttcgaaAAGCTCATCATGCGACG-AAGGTGAGATAGGATTAGG	

Extended Data Table 2 | Construct list. Lowercase font in primer sequence denote adaptor sequence, underlined nucleotides indicate mutations or insertions.

APPENDIX A2

Name	Projects	Sequence 5‘->3‘	Template	Target vector
PUP1_LIC302_F	reporter pPUP1::PUP1-GFP pCB302	tagttggaatgggttcga CTGACTACGCGAGTTCAACAA	genomic DNA	pCB302
PUP1_LIC302_R		tattggagttgggttcga AGCAACATAATCACTAACAGGAAGA	genomic DNA	pCB302
PUP5_LIC302_F	reporter pPUP5::PUP5-GFP pCB302	tagttggaatgggttcga TCGTCGTTCCACAAACTATCAG	genomic DNA	pCB302
PUP5_LIC302_R		tattggagttgggttcga GGATGAAGAAGAGGAAGCTTGA	genomic DNA	pCB302
PUP11_LIC302_F3	reporter pPUP11::PUP11-GFP pCB302	tagttggaatgggttcga TTAATGGATTTCACCCGTGGTATAG	genomic DNA	pCB302
PUP11_LIC302_R		tattggagttgggttcga AAACAACAGGGCGGTTCTACC	genomic DNA	pCB302
PUP12_LIC302_F	reporter pPUP12::PUP12-GFP pCB302	tagttggaatgggttcga ACGGCGTCGTTTAAATAAAGT	genomic DNA	pCB302
PUP12_LIC302_R		tattggagttgggttcga TTCGGCCTGAGCTTCTCGTG	genomic DNA	pCB302
PUP13_LIC302_F	reporter pPUP13::PUP13-GFP pCB302	tagttggaatgggttcga GGTTCTGTTTTTTGTGGGAAGA	genomic DNA	pCB302
PUP13_LIC302_R		tattggagttgggttcga TTCAGCCTGAGCTTCTCGTG	genomic DNA	pCB302
PUP14_LICF-F	reporter pPUP14::PUP14-GFP pCB302	tagttggaatgggttcga GCTTCTGCAGTGAAAGATGTGTT	genomic DNA	pCB302
PUP14_LIC_GFP302_R		tattggagttgggttcgaa TAAGCCATACGATTGTCTTTGTG	genomic DNA	pCB302
PUP15_LIC302_F	reporter pPUP15::PUP15-GFP pCB302	tagttggaatgggttcga AATTCCAAAACTTTGGAAGTTG	genomic DNA	pCB302
PUP15_LIC302_R		tattggagttgggttcgaa TTCGCTTGTGGATTGGTCATCATC	genomic DNA	pCB302
PUP18_LIC_GFP302_F	reporter pPUP18::PUP18-GFP pCB302	tagttggaatgggttcga ATTGTGGTCCAAAATTTCATAC	genomic DNA	pCB302
PUP18_LIC_GFP302_R		tattggagttgggttcgaa GTCAATATTGTTCTCTGTTTG	genomic DNA	pCB302
PUP21_LIC302_F	reporter pPUP21::PUP21-GFP pCB302	tagttggaatgggttcga TCGTTTAAACTTGGGTTCATAGC	genomic DNA	pCB302
PUP21_LIC302_R		tattggagttgggttcga TTTTGATTGCTCACTTGACCC	genomic DNA	pCB302
PUP23_LIC302_F	reporter pPUP23::PUP23-GFP pCB302	tagttggaatgggttcga GGTTAACCTGGGTTCACAAGTC	genomic DNA	pCB302
PUP23_LIC302_R		tattggagttgggttcga AACTTCTACATTGTTCTCACTTTGG	genomic DNA	pCB302
PUP1_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaa ACAGCAAGCAGCAAGAAGAA	genomic DNA, PUP1 pCB302	hbt, DM7, pPLV26
PUP1_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa AGCAACATAATCACTAACAGGAAG	genomic DNA	hbt, DM7
PUP4_LIC_F	ETPamiR, subcellular localization, overexpression, GFP-tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaaACCATCAACGCTTTTTCGA-CACTTC	genomic DNA	hbt, DM7, pPLV26
PUP4_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaaGCCCTATCATCCGCCGCC	genomic DNA	hbt, DM7
PUP10_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcga ATGACGGCGGATCAAGAACTAC	genomic DNA, PUP10 pCB302	hbt, DM7, pPLV26
PUP10_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcga A TTTTGATTGCCAAGTTGACTCTTC	genomic DNA	hbt, DM7
PUP11_LIC_F2	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcga ATTTTCCGATCCCGATTATAAGATG	genomic DNA, PUP11 pCB302	hbt, DM7, pPLV26
PUP11_LIC_R2	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa TGAACAGGGCGGTTCTACCC	genomic DNA	hbt, DM7
PUP14_LIC_F	ETPamiR, subcellular localization, overexpression, GFP-tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcga ATCCATGGCTCAGAATCAACAAC	genomic DNA	hbt, DM7, pPLV26
PUP14_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa ATAAGCCATACGATTGTCTTTGTG	genomic DNA	hbt, DM7
PUP15_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaa ATCAACCAATGCAGTCGTCA	genomic DNA, PUP15 pCB302	hbt, DM7, pPLV26
PUP15_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa GCTTGTGGATTGGTCATCAT	genomic DNA	hbt, DM7
PUP18_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaa CAAATCTTTAAAAGTTGAGAC-CAGAA	genomic DNA, PUP18 pCB302	hbt, DM7, pPLV26
PUP18_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa AACGTCAATATTGTTCTCTGTTTGG	genomic DNA	hbt, DM7
PUP21_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaa TCGAAGGAGGGGGATTCCA	genomic DNA, PUP21 pCB302	hbt, DM7, pPLV26
PUP21_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa TTTTGATTGCTCACTTGACCCTT	genomic DNA	hbt, DM7
PUP23_LIC_F	subcellular localization, overexpression, GFP- tagged overexpression (inducible or constitutive), SDM	tagttggaatgggttcgaa CAGAGAAAACTCATGGAGAT-GACC	genomic DNA, PUP23 pCB302	hbt, DM7, pPLV26
PUP23_LIC_R	ETPamiR, subcellular localization, overexpression, SDM	ttatggagttgggttcgaa AACTTCTACATTGTTCTCACTTTGG	genomic DNA	hbt, DM7
GFP_LIC_R	GFP- tagged overexpression (inducible or constitutive)	tattggagttgggttcgaTTACTTGTACAGCTCGTCCATGC	PUP pCB302	DM7, pPLV26

Appendix Table 1 | Primer list (1/3) (continued on next pages). LIC-adaptor sequences are indicated in lowercase letters. Target codons for site-directed mutagenesis are indicated in bold. Antisense and sense *amiR* sequences are underlined.

Name	Projects	Sequence 5‘->3‘	Template	Target vector
PUP14_P100G_F	SDM	TGTTGGTTTC**GGT**CTCCTTC	genomic DNA, PUP14 pCB302	DM7
PUP14_P100G_R		GAAGGAG**ACC**GAAACCAACA	genomic DNA, PUP14 pCB302	DM7
PUP14_Q319A_F		TTCGTGG**GCG**GTCTATTG	genomic DNA, PUP14 pCB302	DM7
PUP14_Q319A_R		CAATAGAC**CGC**CCACGAA	genomic DNA, PUP14 pCB302	DM7
PUP14_S333C_F		GTCTACTCTGTT**TGC**AGCGTG	genomic DNA, PUP14 pCB302	DM7
PUP14_S333C_R		CACGCT**GCA**AACAGAGTAGAC	genomic DNA, PUP14 pCB302	DM7
PUP14_S333E_F		TGTCTACTCTGTT**GAG**AGCGTGT	genomic DNA, PUP14 pCB302	DM7
PUP14_S333E_R		ACACGCT**CTC**AACAGAGTAGACA	genomic DNA, PUP14 pCB302	DM7
PUP10_P88H_F		GTTGGCTTT**CAT**GTGCTACTTC	genomic DNA, PUP10 pCB302	DM7
PUP10_P88H_R		GAAGTAGCAC**ATG**AAAGCCAAC	genomic DNA, PUP10 pCB302	DM7
PUP10_Q295A_F		CCTGG**GCG**GTATTCTCCATCG	genomic DNA, PUP10 pCB302	DM7
PUP10_Q295A_R		CGATGGAGAATAC**CGC**CCAGG	genomic DNA, PUP10 pCB302	DM7
PUP10_S309A_F		GCTC**GCC**TCTCTATTCTCAAATGC	genomic DNA, PUP10 pCB302	DM7
PUP10_S309A_R		GCATTTGAGAATAGAGA**GGC**GAGC	genomic DNA, PUP10 pCB302	DM7
PUP10_S309E_F		GCTC**GAG**TCTCTATTCTCAAATGC	genomic DNA, PUP10 pCB302	DM7
PUP10_S309E_R		GCATTTGAGAATAGAGA**CTC**GAGC	genomic DNA, PUP10 pCB302	DM7
PUP11_P87L_F		CGGCTGCTTTT**GTG**ATACTCTA	genomic DNA, PUP11 pCB302	DM7
PUP11_P87L_R		TAGAGTAT**CAC**AAAAGCAGCCG	genomic DNA, PUP11 pCB302	DM7
PUP11_Q293A_F		TGG**GCA**GTGTGTTCTGTTG	genomic DNA, PUP11 pCB302	DM7
PUP11_Q293A_R		CAACAGAACACAC**TGC**CCA	genomic DNA, PUP11 pCB302	DM7
PUP11_T307C_F		TCTGGTG**TGC**TCGCTCTTC	genomic DNA, PUP11 pCB302	DM7
PUP11_T307C_R		GAAGAGCGA**GCA**CACCAGA	genomic DNA, PUP11 pCB302	DM7
PUP11_T307E_F		TCTGGTG**GAG**TCGCTCTTC	genomic DNA, PUP11 pCB302	DM7
PUP11_T307E_R		GAAGAGCGA**CTC**CACCAGA	genomic DNA, PUP11 pCB302	DM7
PUP18_P92A_F		ACGCTGCGTTT**GCA**CTACT	genomic DNA, PUP18 pCB302	DM7
PUP18_P92A_R		AGTAG**TGC**AAACGCAGCGT	genomic DNA, PUP18 pCB302	DM7
PUP18_Q304A_F		CATTGTCGTGG**GCG**GTTT	genomic DNA, PUP18 pCB302, PUP23 pCB302	DM7
PUP18_Q304A_R		AAAC**CGC**CCACGACAATG	genomic DNA, PUP18 pCB302, PUP23 pCB302	DM7
PUP18_S318A_F		GCTTTATGTT**TGC**GGTGTGT	genomic DNA, PUP18 pCB302	DM7
PUP18_S318A_R		ACACACC**GCA**AACATAAAGC	genomic DNA, PUP18 pCB302	DM7
PUP18_S318E_F		TGCTTTATGTT**GAG**GGTGTGT	genomic DNA, PUP18 pCB302	DM7
PUP18_S318E_R		ACACACC**CTC**AACATAAAGCA	genomic DNA, PUP18 pCB302	DM7
PUP23_P96A_F		ACGCTGCTTTT**GCA**ATCCT	genomic DNA, PUP23 pCB302	DM7
PUP23_P96A_R		AGGAT**TGC**AAAAGCAGCGT	genomic DNA, PUP23 pCB302	DM7
PUP23_S322A_F		GCTTTATGTT**TGC**AGTGTTT	genomic DNA, PUP23 pCB302	DM7
PUP23_S322A_R		AAACACT**GCA**AACATAAAGC	genomic DNA, PUP23 pCB302	DM7
PUP23_S322E_F		GCTTTATGTT**GAG**AGTGTTT	genomic DNA, PUP23 pCB302	DM7
PUP23_S322E_R		AAACACT**CTC**AACATAAAGC	genomic DNA, PUP23 pCB302	DM7
amiR_PUP4_1_I	amiR PUP4_1	GATAAGAAGTGTCGAAAAAGCGCTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP4_1_II		GAGCGCTTTTTCGACACTTCTTATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP4_1_III		GAGCACTTTTTCGACTCTTCTTTTCACAGGTCGT-GATATG	pRS3000	DM7
amiR_PUP4_1_IV		GAAAAGAAGAGTCGAAAAAGTGCTCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP4_2_I	amiR PUP4_2	GATATAATCATAACGGCCGTCTGTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP4_2_II		GACAGACGGCCGTTATGATTATATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP4_2_III		GACAAACGGCCGTTAAGATTATTTCACAGGTCGT-GATATG	pRS3000	DM7
amiR_PUP4_2_IV		GAAATAATCTTAACGGCCGTTTGTCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP4_3_I	amiR PUP4_3	GATGATCGTGGATACATACGCGATCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP4_3_II		GATCGCGTATGTATCCACGATCATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP4_3_III		GATCACGTATGTATCGACGATCTTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_PUP4_3_IV		GAAGATCGTCGATACATACGTGATCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP11_1_I	amiR PUP11_1	GATATCATACATTCGTACGGCACTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP11_1_II		GAGTGCCGTACGAATGTATGATATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP11_1_III		GAGTACCGTACGAATCTATGATTTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_PUP11_1_IV		GAAATCATAGATTCGTACGGTACTCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP11_2_I	amiR PUP11_2	GATTTGATCATACATTCGTGCGATCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP11_2_II		GATCGCACGAATGTATGATCAAATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP11_2_III		GATCACACGAATGTAAGATCAATTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_PUP11_2_IV		GAATTGATCTTACATTCGTGTGATCTACATA-TATATTCCT	pRS3000	DM7

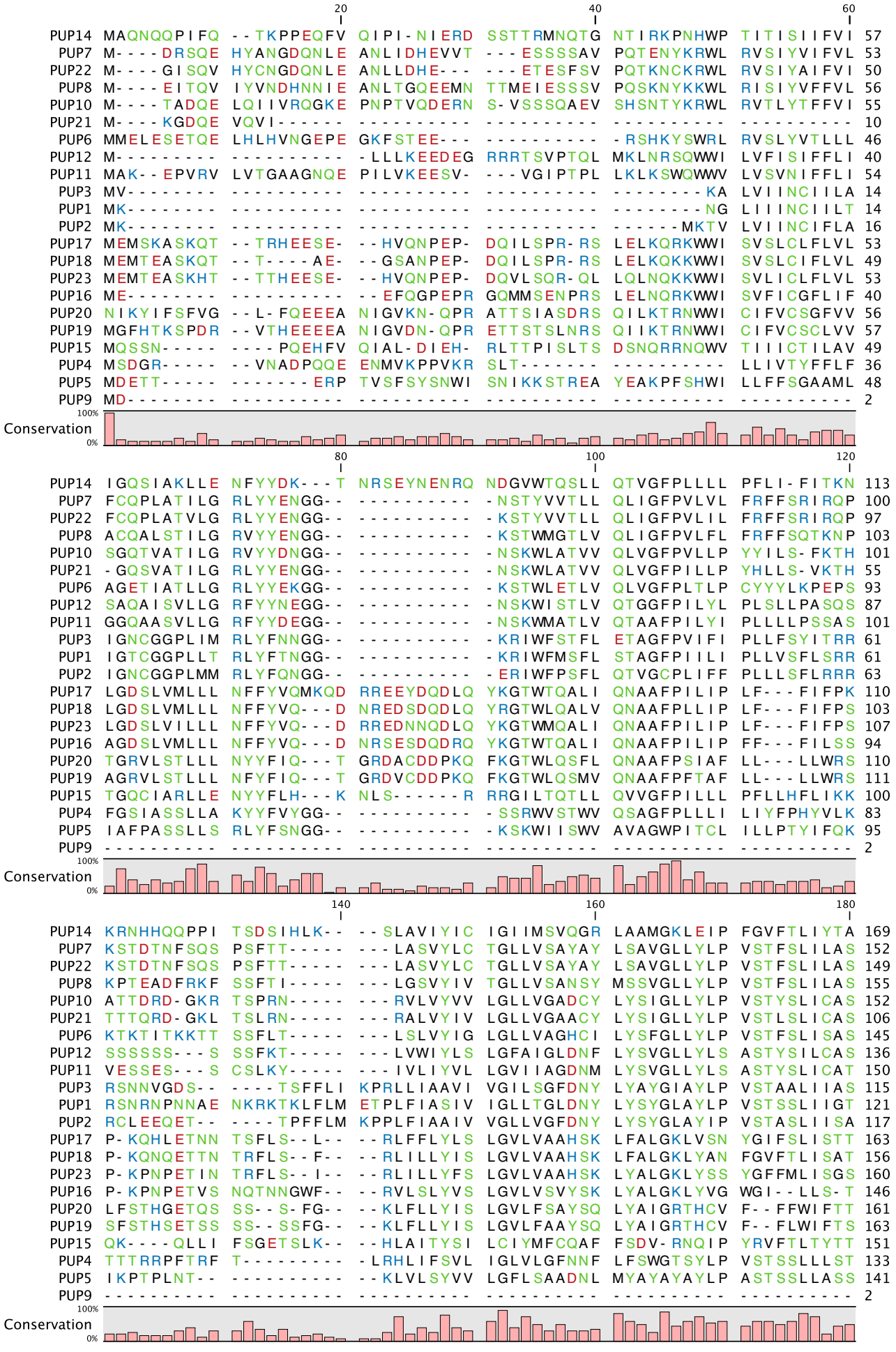
Appendix Table 1 | Primer list (continued 2/3). PUP18_Q304A primers were also used for PUP23 mutagenesis.

Name	Projects	Sequence 5‘-> 3‘	Template	Target vector
amiR_PUP11_3_I	amiR PUP11_3	GATATCATACATTCGTACGACAGTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP11_3_II		GACTGTCGTACGAATGTATGATATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP11_3_III		GACTATCGTACGAATCTATGATTTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_PUP11_3_IV		GAAATCATAGATTCGTACGATAGTCTACATA-TATATTCCT	pRS3000	DM7
amiR_A_I	amiR14_1	GATTATTTGCACAAAGTGTTCTGTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_A_II		GACAGAACACTTTGTGCAAATAATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_A_III		GACAAAACACTTTGTCCAAATATTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_A_IV		GAATATTTGGACAAAGTGTTTTGTCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP14_2_I	amiR14_2	GATGTTGATAGGTATTTGCACGATCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP14_2_II		GATCGTGCAAATACCTATCAACATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP14_2_III		GATCATGCAAATACCAATCAACTTCACAGGTCGTGA-TATG	pRS3000	DM7
amiR_PUP14_2_IV		GAAGTTGATTGGTATTTGCATGATCTACATA-TATATTCCT	pRS3000	DM7
amiR_PUP14_3_I	amiR14_3	GATAGTTCAATGTATGTCGGCGCTCTCTCTTT-TGTATTCC	pRS3000	DM7
amiR_PUP14_3_II		GAGCGCCGACATACATTGAACTATCAAAGAGAAT-CAATGA	pRS3000	DM7
amiR_PUP14_3_III		GAGCACCGACATACAATGAACTTTCACAGGTCGT-GATATG	pRS3000	DM7
amiR_PUP14_3_IV		GAAAGTTCATTGTATGTCGGTGCTCTACATA-TATATTCCT	pRS3000	DM7
LIC-OLIGO_A	amiRs	tagttggaatgggttcgaa CGACGTTGTAAAACGACGGCCAG	pRS3000	DM7
LIC-OLIGO_B		ttatggagttgggttcgaa CTCGGAATTAACCCTCACTAAAGG	pRS3000	DM7
AS73 116	genotyping Δ*pup11* N348765 GK-421B11.01	ATATTGACCATCATACTCATTGC	genomic DNA	
AS73 356		ACATGTTAAGAGGCCCAAACC		
AS73 357		GTGACGTCGCTCTTCTCAAAC		
AS85 109	genotyping Δ*pup11* N661092 SALK_112079C	ATTTTGCCGATTTCGGAAC		
AS85 315		GAAGCAAAACCAGAAGGAAAG		
AS85 316		TGATGATGCGGATACTCCTTC		
GK348G04_RP2	genotyping Δ*pup4* N321554	TCATAACGGCCGTCTCAATCAC		

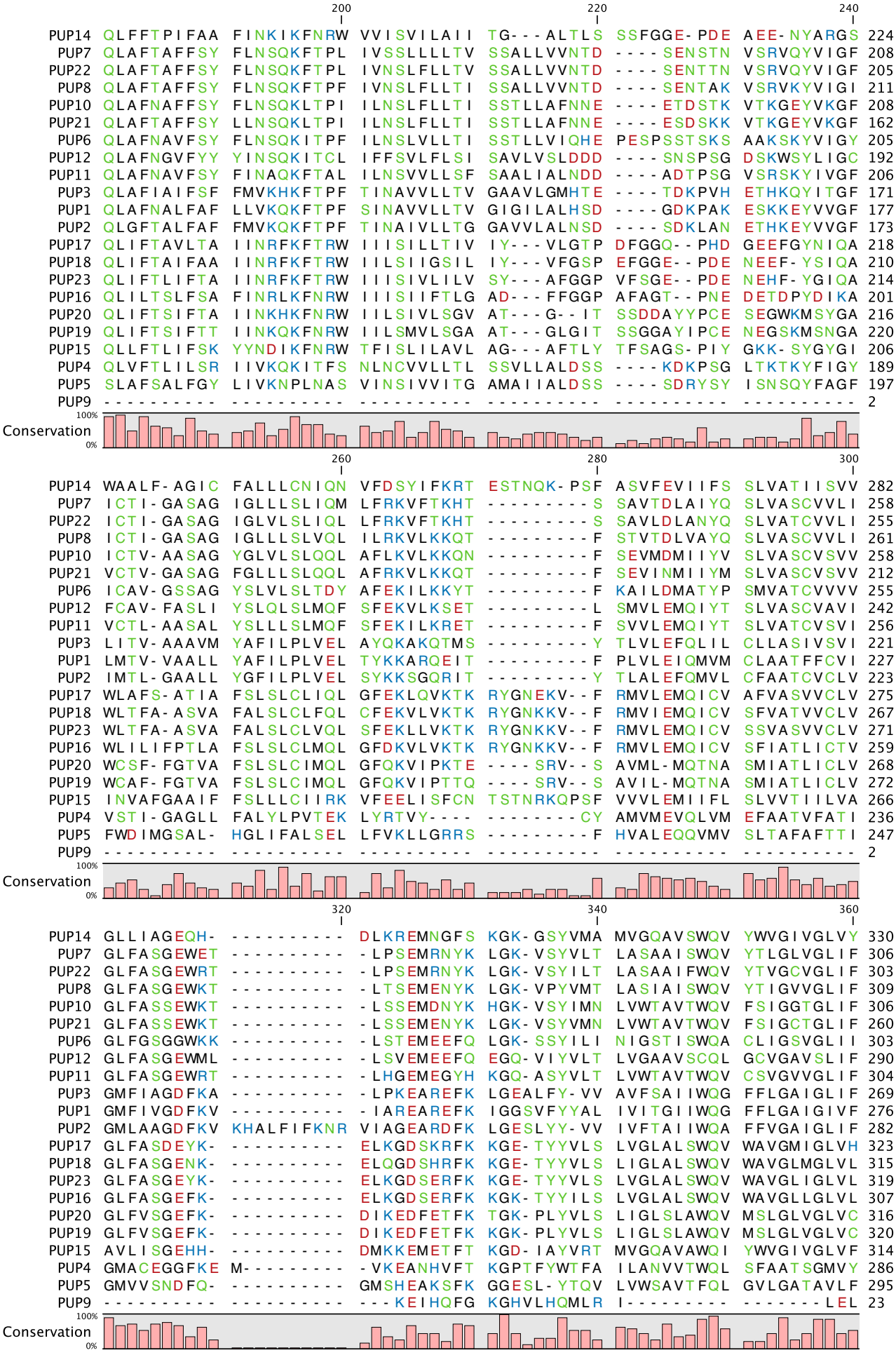
Appendix Table 1 | Primer list (continued 3/3). LIC-adaptor sequences are indicated in lowercase letters. Target codons for site-directed mutagenesis are indicated in bold. Antisense and sense *amiR* sequences are underlined.

		hbt	*HA*	*GFP*	**pCB302**	*vector*	*plants*	**DM7**	*DM7 in TCSn (plants)*	*GFP DM7 (vector)*	*GFP DM7 plant*	**pPLV26**	*vector*	*plants*	**Mutants**	*amiR target*	*MIGS*	*T-DNA line*	**SDM DM7**	*P*	*Q*	*S->C*	*S->E*	**SDM GFP DM7**	*P*	*Q*	*S->C*	*S->E*
PUP1	AT1G28230		x	a		a	a		a	x			a	a				a											
PUP2	AT2G33750																												
PUP3	AT1G28220															a													
PUP4	AT1G30840		a	a		a	a		a	a	x		a	a				a											
PUP5	AT2G24220					a	a																						
PUP6	AT4G18190																												
PUP7	AT4G18197																												
PUP8	AT4G18195																												
PUP9	AT1G18220																												
PUP10	AT4G18210		a	a					a	x			a	a		a				o	o	o	o		o	o	v	v
PUP11	AT1G44750		a	a		a	a		a	x								a											
PUP12	AT5G41160					a	a									a													
PUP13	AT4G08700					a	a									a													
PUP14	AT1G19770		a	a		a	a		a	a	a		a	x		a	a			p	p	p	p		o	o	o	v
PUP15	AT1G75470		a	a		a	a		a	a	a		a	a		a													
PUP16	AT1G09860																												
PUP17	AT1G57943															a													
PUP18	AT1G57990		a	a		a	a		a	a	a		a	a		a	a			v	p	p	p		o	v	v	o
PUP19	AT1G47603															a													
PUP20	AT1G47590															a													
PUP21	AT4G18220		a	a		a	a		a	a	a					a		a											
PUP22	AT4G18205																												
PUP23	AT1G57980			a		a	a		a	a	a		a	a						v	p	p	v		v	o	v	v

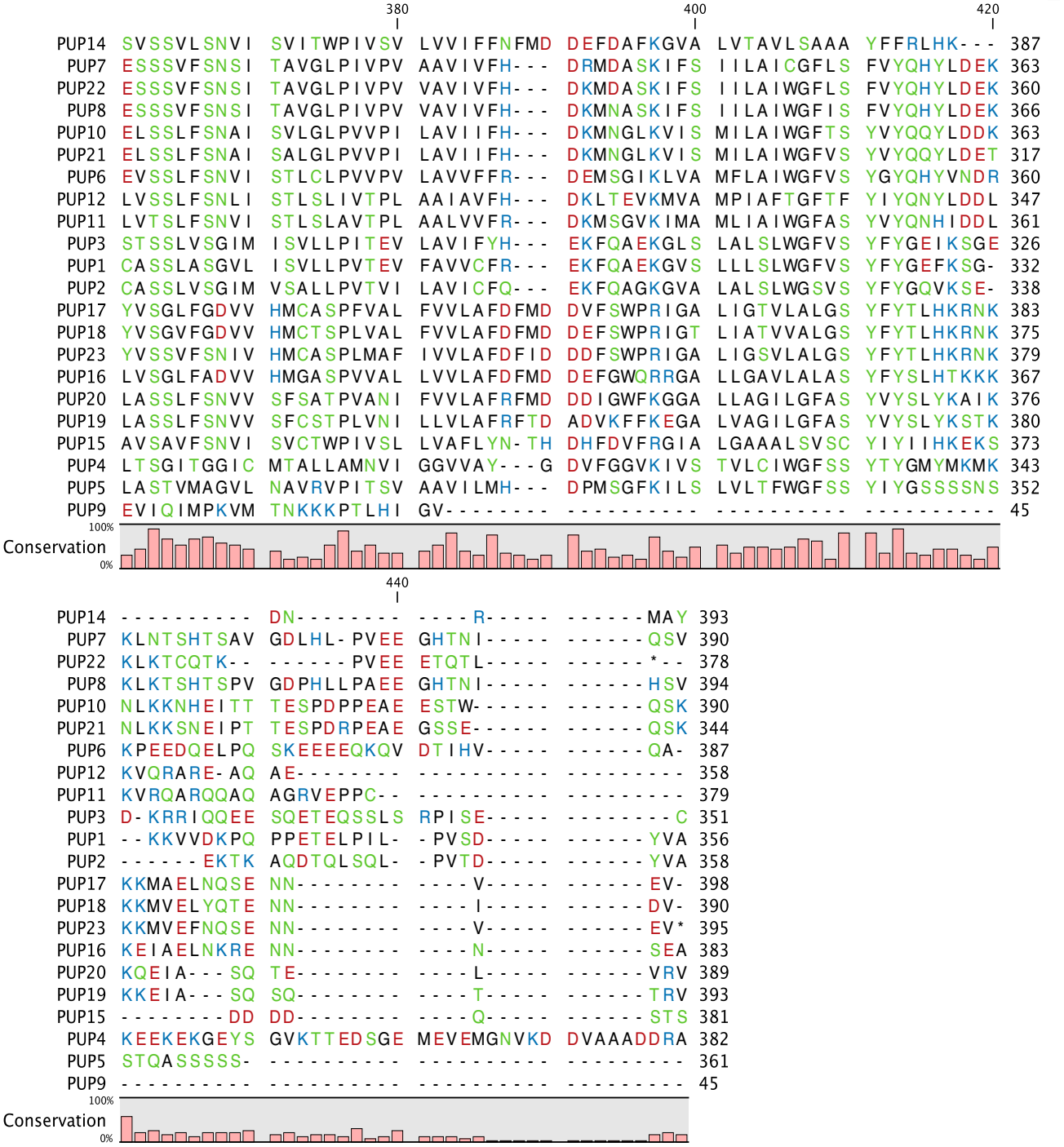
Appendix Table 2 | Constructs. a = available; x = cloning failed or no positive transformants; o = cloning ongoing; v = vector available; p = plant available (o, v, p valid only for mutants)



Appendix Figure 1 | PUP peptide alignment (1/3) (continued on next pages).



Appendix Figure 1 | PUP peptide alignment (2/3).



Appendix Figure 1 | PUP peptide alignment (3/3).